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METABOLIC EFFECTS OF HUMAN GROWTH HORMONE AND GROWTH HORMONE OF OTHER SPECIES IN MAN

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ABSTRACT

The metabolic effects of human growth hormone were investigated under varied clinical conditions in 15 balance studies on 10 subjects. Nitrogen retention occurred uniformly, the amount varying from 2 to 4 Gm. daily. Potassium and phosphorus were likewise retained, but in over half the studies, in amounts greater than those calculated from the nitrogen retention. This suggested the deposition of parenchymal tissue rather than muscle tissue. Hypercalciuria and sodium chloride retention often accompanied the administration of human growth hormone. Growth hormone was effective in the hypothyroid subject. Neither increased nor decreased adrenal function altered the anabolic effect. Sheep and whale growth hormone were metabolically inactive in single trials. Nine studies with chymotrypsinized beef growth hormone likewise failed to indicate anabolic activity.

THE anabolic effects of growth hormone have been appreciated since the pioneering studies of Evans and Long (1). However, fruitful investigation of this phenomenon in man is of recent origin and there are relatively few reports about the use of human growth hormone (HGH) in man. We have examined the response to HGH in patients presenting a number of clinical conditions in an attempt to delineate some of the factors affecting this response.

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Detailed descriptions of the changes in nitrogen and mineral metabolism in man resulting from administration of HGH have been presented (2–4). It is not the purpose of this report to confirm extensively these findings, but rather to summarize our own experiences. For this reason, the metabolic data have been presented in summary form.

The suggestion that growth hormone demonstrates species specificity (5, 6), and the subsequent proof of this in the monkey (7, 8), made it apparent that only limited supplies of a growth hormone effective in man could be obtained from primate sources. The determination of the structure of HGH (9) stimulated attempts to obtain an active fraction from beef growth hormone by partial enzymatic hydrolysis (10). We have extensively tested such a product, reported to be active (11), but have been unable to demonstrate effectiveness in man. Growth hormone derived from sheep and whale pituitaries has likewise been examined in the hope of finding a growth hormone with anabolic properties in man.

METHODS

Human growth hormone was isolated from glands obtained at autopsy. Various batches of HGH were prepared by Dr. C. H. Li, Berkeley, California (9); Dr. M. J. Raben, Boston, Massachusetts (12); and Dr. N. Brink, of Merck and Company, Rahway, New Jersey (13). The material was given intramuscularly in fractional doses. It was injected at pH 8.5 (Li), pH 3.0–3.5 (Raben), and at a neutral pH (Merck). The chymotrypsinized beef growth hormone (11), the sheep growth hormone (14) and the whale growth hormone (15) were prepared by Dr. C. H. Li by the indicated methods. Each lot of growth hormone was bioassayed by the rat weight-gain method or by the stimulation of S^{35}-uptake in rat cartilage (16).

All of the patients were studied using standard metabolic balance techniques. The theoretical potassium retention and phosphorus retention were calculated using a K/N ratio of 3/1 and an N/P ratio of 15/1 (17). The analytical methods used in this laboratory have been previously reported (18).

Pertinent details about the patients and the dose of growth hormone are listed in Table 1. The patients with carcinoma of the breast and choriocarcinoma were in a state of remission during the study. Panhypopituitarism was clearly indicated in Patients K.S. and M.S. by the demonstration of thyroidal 24-hour I^{131} uptakes below 10 per cent, serum protein-bound iodine levels of less than 3 μg. per 100 ml., and 24-hour excretions of 17-hydroxycorticoids below 2 mg. Patient A.S. with adrenal carcinoma had an elevated urinary excretion of 17-ketosteroids and an abdominal mass, but showed no other systemic effects of the carcinoma.

RESULTS

In all 15 studies, the administration of HGH resulted in nitrogen retention (Table 2). This anabolic effect was manifested by retentions ranging from 0.5 Gm. to 3.8 Gm. per twenty-four hours. Nitrogen retention often was apparent on the first day of administration of HGH, and continued

TABLE 1. SUBJECTS AND PROTOCOLS

Patient	Sex and age	Weight (Kg.)	HGH, daily dose (mg.) and type*	Duration of therapy (days)	Other hormones**	Nitrogen intake (Gm./24 hrs.)	Calories per 24 hrs.	Diagnosis and status
E.Ga.	F 34	41.5	5-L	6	F, 10 mg. T_3, 25 μg.	10.6	1900	Sheehan's syndrome
W.C.	M 64	78.4	10-L	6	F, 10 mg. T_3, 25 μg.	13.2	2145	Chromophobe adenoma;
		80.8	5-L	5	25 μg.	8.7	2076	panhypopituitarism
		80.8	5-R	5	25 μg.	8.7	2076	
		81.2	10-M	6	25 μg.	9.9	2112	
K.S.	F 34	73.0	5-R	10		11.3	1711	Chromophobe adenoma;
		70.6	5-R	6	F, 30 mg. T_3, 50 μg.	11.3	1689	panhypopituitarism
		69.9	5-M	4	50 μg.	11.3	1689	
M.S.	F 26	45.1	5-R	6		10.3	2546	Hypophyseal stalk section; choriocarcinoma
E.G.	F 38	55.0	5-L	6	F, 20 mg. T_3, 75 μg.	10.6 10.6	2122	Hypophysectomy; breast cancer
N.M.	F 48	44.3	5-L	5		9.1	1724	Ovariectomy; breast cancer
C.M.	F 50	49.5	5-M	6	F, 30 mg. T_3, 50 μg.	9.5	1650	Hypophysectomy; breast cancer
E.Gi.	F 38	58.2	5-L	6	F, 30 mg. T_3, 25 μg.	12.7	1896	Hypophysectomy; breast cancer
A.S.	M 49	71.1	5-R	6		14.7	2493	Adrenal carcinoma
E.G.	F 34	59.6	5-R	6		9.9	1818	Cushing's syndrome; adrenal hyperplasia
M.L.	M 39	80.0	5-M	7		13.3	2340	Normal

* L—Prepared by C. H. Li.
R—Prepared by M. S. Raben.
M—Prepared by N. Brink (Merck).
** F—Hydrocortisone.
T_3—Triiodothyronine.

TABLE 2. BALANCE DATA

Patient	Balance												Blood urea nitrogen (mg./100 ml.)	
	Nitrogen (Gm./24 hrs.)			Phosphorus (Gm/24 hrs.)			Potassium (mEq./24 hrs.)			Sodium (mEq./24 hrs.)				
	C*	HGH	C	C	HGH	C	C	HGH	C	C	HGH	C	C	HGH
W.C., 1	+1.1	+3.2	+0.7	+0.26	+0.31	+0.17	−2	+12	+1	+5	+22	− 3	11	5
2	−0.6	+1.5	−0.8	−0.02	+0.11	+0.04	+3	+17	−1	+8	+ 9	−11	12	9
3	−0.3	+2.3	−0.4	−0.24	+0.51	+0.12	−2	+23	−4	+2	+17	− 5	12	8
1	+0.6	+2.5	−0.1	+0.07	+0.49	+0.05	+3	+26	+4	+3	+31	−16	14	12
K.S., 1	+0.2	+2.9	−0.4	+0.05	+0.37	−0.02	+4	+12	−3	+1	+24	−11	15	7
2	−0.1	+3.2	−0.2	−0.11	+0.03	−0.14	−2	+14	+2	+3	+14	−16	13	9
3	−0.3	+3.3		−0.08	+0.12		+1	+ 2		+6	+27		12	3
M.S.	+1.4	+2.3	+1.7	+0.12	+0.34	+0.25	+14	+22	+12	+7	+ 6	−14	7	8
E.Ga.	−1.5	+0.5	−1.3	−0.24	−0.21	−0.20	−3	+ 4	+1	−1	+ 2	+ 4	9	4
N.M.**	+0.4	+2.2	+0.6							+2	+ 4	+ 4	11	11
C.M.	−0.5	+2.0	−0.6	−0.04	+0.71	−0.52	+7	+15	+2	+3	+33	−25	14	9
E.Gi.	−0.8	+0.5	+0.2	+0.18	+0.19	+0.14	−13	+ 3	−5	−9	+ 1	−11	8	5
A.S.	+0.1	+2.2	−1.6	+0.03	+0.14	+0.01	+3	+ 8	+4	0	+ 1	− 4	18	10
E.Gr.	−0.3	+2.7	−0.8	+0.08	+0.22	+0.02	+2	+14	−4	−4	+ 1	− 6	12	8
M.L.**	−0.1	+3.8	−0.2							+5	+16	+ 2	13	12

* C = Control.
** Stool analyses not performed. The nitrogen and sodium balances were calculated assuming a daily fecal excretion of 1.0 Gm. of nitrogen and 1 mEq. of sodium.

for one to six days after HGH had been discontinued. In these 15 studies, HGH decreased the urinary nitrogen by an average of 2.3 Gm. daily. A decrease in the level of blood urea nitrogen generally accompanied the nitrogen retention.

An increase in urinary calcium occurred as a result of HGH administration and has been reported in detail (19). This increase varied from zero to 164 mg. per twenty-four hours. There were no consistent changes in fecal calcium excretion during any of the studies. Since the balance periods and the period of HGH administration were short, it seemed inadvisable to

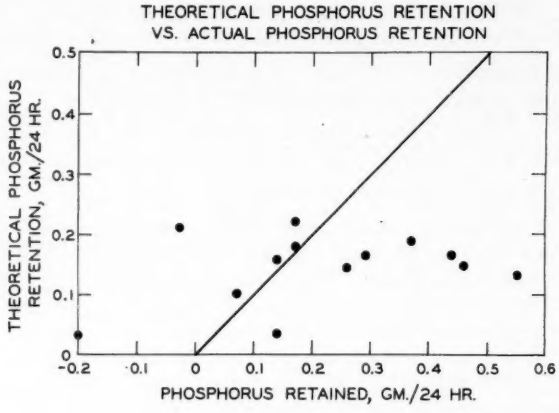

FIGURE 1

attempt to characterize completely the effect of HGH on calcium balance. For purposes of calculation of theoretical phosphorus balances, however, it was assumed that the changes in urinary calcium excretion during the test periods represented the only alteration in calcium balance.

Phosphorus retention almost uniformly accompanied nitrogen retention. The amount retained often exceeded that calculated from the nitrogen retention and the changes in urinary calcium excretion. In Figure 1, the theoretical phosphorus retention was plotted against the actual phosphorus retention. Seven of the points were below the line of agreement, 4 were on the line, and in only 2 studies was the phosphorus retention less than that calculated.

Similarly, potassium retention accompanied nitrogen storage. When actual potassium retention was plotted against expected potassium retention (Fig. 2), it was noted that more potassium was retained than expected in 12 of the 13 studies.

Retention of sodium and chloride was observed in 8 of the 15 studies. Only the sodium balance data were recorded, since the chloride balance

data ran parallel. Sodium retention was as high as 33 mEq. in twenty-four hours. This retention did not always continue throughout the period of HGH administration, and occasionally a spontaneous natriuresis occurred during the test period.

The serum concentration of calcium, phosphorus and alkaline phosphatase remained normal. The urinary excretion of α-amino acid nitrogen, 17-ketosteroids and 17-hydroxycorticoids was not affected by HGH.

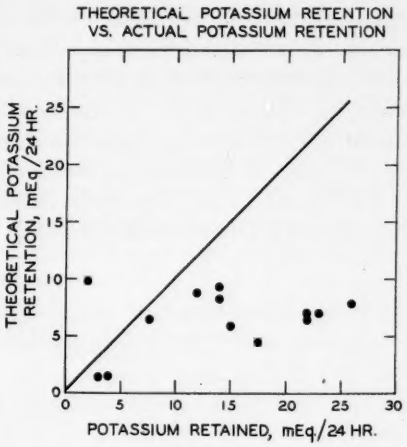

FIGURE 2

Three of the studies permitted comparison of the three preparations of HGH. One of these is shown in Figure 3. In the first and fourth study on Patient W.C. (Table 2), the comparison of HGH-L(Li) with HGH-M (Merck) revealed that each caused retention of approximately

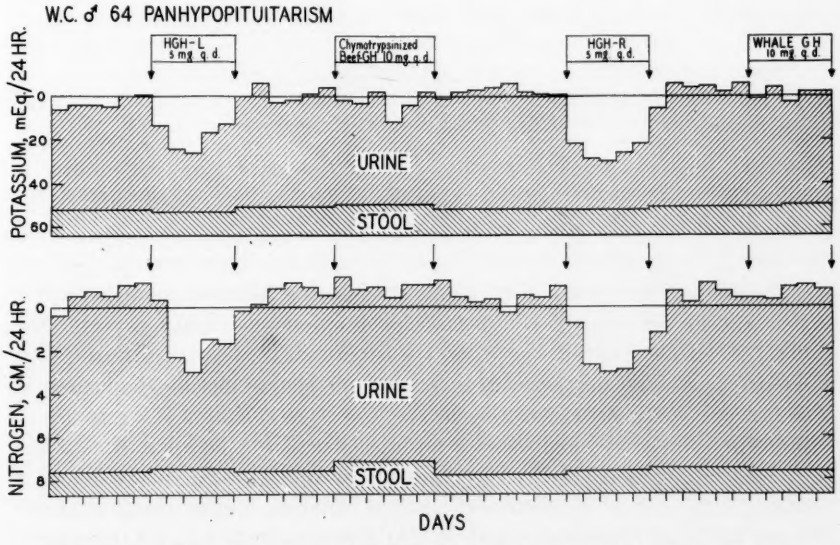

FIG. 3. Comparison of the effects of HGH-Li, chymotrypsinized beef growth hormone HGH-Raben, and whale growth hormone on potassium and nitrogen metabolism in Patient W.C. In Figures 3 and 4, the nitrogen intake is plotted from the zero line down. Superimposed on this are the values for urinary and fecal nitrogen. A positive balance is represented by a clear space below the zero line.

equal amounts of nitrogen. The second and third studies on Patient W.C. (Table 2) permitted the comparison of HGH-L and HGH-R (Raben) and demonstrated that each preparation had about the same potency. Similarly, in Patient K.S., HGH-R and HGH-M were shown to be equally potent. There were no apparent differences among the three preparations with respect to tolerance or metabolic effects.

In Patient E.Ga., the effectiveness of the intravenous route for administration of HGH was analyzed (Fig. 4). The HGH was given in an infusion

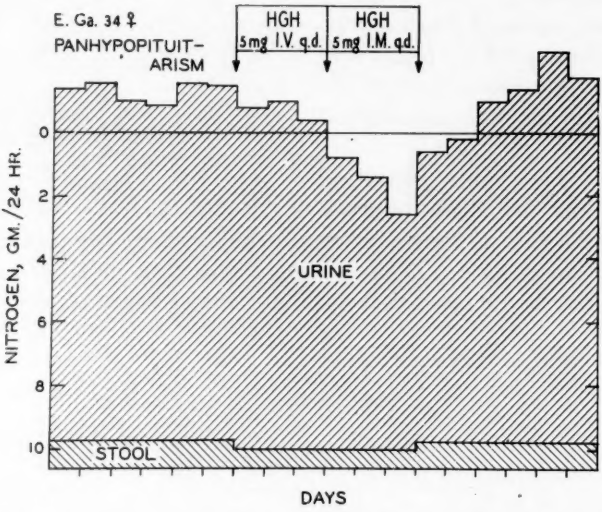

FIG. 4. Intravenous route versus intramuscular route of HGH administration; effect on nitrogen retention in Patient E.Ga.

of physiologic saline twice daily over eight-hour periods. Nitrogen retention was questionable during the period of intravenous infusion, but subsequently occurred promptly when the HGH was given intramuscularly. Since nitrogen retention was observed for only three days, calculations of N/P and K/N ratios were not made.

HGH exhibited its anabolic effect under varying conditions. Patient K.S. was clinically hypothyroid, and there was laboratory evidence of marked depression of thyroid and adrenal function during the first study. Yet the nitrogen retention noted in this deficient state was 90 per cent of that noted following adrenal and thyroid replacement therapy. Similarly, HGH was effective in Patient M.S., although thyroid and adrenal replacement therapy had been withheld.

In Patient E.Gr., who received an excess of hydrocortisone therapy, HGH was likewise an effective anabolic agent.

Growth hormone derived from the Macaca rhesus monkey was tested at a dosage level of 5 mg. daily in Patient W.C. It produced nitrogen retention of the same magnitude as that obtained with 5 mg. of HGH-L. Because whale growth hormone has a molecular weight intermediate between bovine and human growth hormone (15), one study was performed with this material (Fig. 4); it proved to be inactive. Sheep growth hormone was also tested because of the finding that ovine prolactin is an anabolic agent (20); this growth hormone was likewise inactive.

Nine subjects received bovine growth hormone that had been exposed to chymotrypsin in an attempt to produce a growth factor that would be active in man. Four of these patients (K.S., E.Ga., E.Gi. and W.C.) had been shown to be responsive to HGH. Two other adults and 3 children, one of whom had gonadal dysgenesis, also received this material. In no instance was an anabolic effect obtained. There were no detectable metabolic effects; the calcium and sodium balances remained stationary.

DISCUSSION

The striking feature of these and other studies (2, 3, 4, 21) with HGH has been the almost uniform retention of nitrogen noted in the adult. The amount of nitrogen retained has varied between 2 and 4 Gm. daily and only rarely has the daily nitrogen retention been less than 2 Gm. In treatment periods as long as sixteen days (4), there was no tendency towards a decreasing anabolic effect.

The retention of potassium in amounts significantly greater than can be accounted for on the basis of nitrogen retention was also noted by Ikkos *et al.* (4). In another report (3), potassium retention was said to be proportional to nitrogen retention, although examination of Figure 1b in this report suggests excessive potassium retention. These data appear more impressive in view of the fact that there was often concomitant sodium retention which, *per se*, tends to cause potassium loss. A possible explanation of this phenomenon is that the tissue deposited under the influence of HGH is not muscle, but rather parenchymal tissue. In parenchymal tissue, particularly liver, the K/N ratio tends to be higher than in muscle. In support of this hypothesis, one can cite the disproportionate visceromegaly of acromegaly.

The relationship of phosphorus balance to nitrogen balance was not as consistent. In 4 of the studies, phosphorus was retained in proportion to nitrogen. This is in agreement with the data of other reports. However, in 7 of the tests, there was excessive retention of phosphorus—again suggesting an increase of parenchymal tissue with a relatively high P/N ratio.

Retention of sodium and chloride has been observed in the adrenalecto-

mized rat given growth hormone (22), and also in normal man (2, 4). The observed increase in extracellular fluid volume of the acromegalic (23) is presumably a reflection of this condition. The mechanisms of this phenomenon are unknown. Only occasionally has the sodium retention been accompanied by increased aldosterone secretion (2), and in one series a rise in aldosterone secretion was not observed (4).

During the early studies with growth hormone, it was observed that thyroid hormone greatly increased the degree of nitrogen retention promoted by growth hormone in the rat (21). In our 2 studies, HGH was shown to be effective in patients with hypothyroidism, and the addition of triiodothyronine to the regimen in one patient led to questionable enhancement of the anabolic potency of HGH. These data are too few to compare with those of animal studies, so the only conclusion that can be drawn is that HGH is substantially effective in the hypothyroid subject.

An antagonism between HGH and adrenal secretions has been demonstrated in the rat (24). In man, hyperadrenocorticism, manifested as Cushing's syndrome (Patient E.G.), did not negate the response to HGH. Since nitrogen balance was negative in this patient, in spite of a diet and caloric intake of her own choosing, the implication is that endogenous compensatory factors, presumably including growth hormone, were unable to compensate for the catabolic effects of hydrocortisone therapy. Parenthetically, it should be noted that HGH did not influence the excretion of 17-ketosteroids or 17-hydroxycorticoids by this patient with adrenal hyperplasia.

It has been difficult to bring such facets of HGH activity as retention of sodium and chloride and hypercalciuria into harmony with a reasonable scheme of action of HGH. Although there is no acceptable explanation for the salt retention and the hypercalciuria, these phenomena may be of lesser consequence when it is appreciated that they are the effects of pharmacologic amounts of HGH. The degree of nitrogen retention seen in the adult supports this point, as a simple calculation will show. The total amount of nitrogen in an adult male may be estimated at 1.5 Kg. If this entire amount were retained throughout the years of growth and development, then it would be difficult, by the balance technique, to demonstrate a positive nitrogen balance of less than 0.5 Gm. daily. Yet HGH in the doses used results in positive nitrogen balances of 2 to 4 Gm. daily. Thus, the data of this study and other studies reflect the effects of excessive doses of HGH, and such unexpected findings as sodium retention and hypercalciuria may not necessarily be significant features of the effects of *physiologic* amounts of growth hormone.

Because of the demonstration of the species specificity of growth hormone, it was not unexpected that sheep and whale growth hormones were

inactive in man. We did hope, however, that partial digestion of beef growth hormone by chymotrypsin would uncover an "active core." Forsham and co-workers (11) have reported such an effect in several patients. Using an identical preparation, we did not obtain any evidence of anabolism. Since an impressive amount of nitrogen retention was demonstrated by Forsham *et al.* in one patient with gonadal dysgenesis (11), we also attempted to produce a positive nitrogen balance in such a patient with chymotrypsinized beef growth hormone, but were unsuccessful. It has been suggested (personal communication from Dr. C. H. Li) that minor differences in the dilution of the dried powder and the length of time it is allowed to remain in solution before injection may account for these varying results. Only extended work with partially degraded beef growth hormone will resolve these discrepancies.

Acknowledgments

We gratefully acknowledge the invaluable cooperation of Dr. C. H. Li in the preparation of the human, whale, sheep, monkey and chymotrypsinized beef growth hormone. We also wish to thank Dr. M. S. Raben and Dr. N. Brink for their generous assistance in preparing human growth hormone. Misses Jeanne Reid and Alice Stang, dietitians, made these balance studies possible. Mr. Sidney Siegel skillfully conducted the bioassays. The metabolic data were obtained under the direction of Mr. Fred Dhyse. Our resident and nursing staff during the past three years have greatly furthered our efforts by their careful attention to the problems of the metabolic balance patient.

REFERENCES

1. Evans, H. M., and Long, J. A.: The effect of the anterior lobe administered intraperitoneally upon growth, maturity, and oestrous cycles of the rat, *Anat. Rec.* **21**: 62 (Apr.) 1921.
2. Beck, J. C.; McGarry, E. E.; Dyrenfurth, I., and Venning, E. H.: The metabolic effects of human and monkey growth hormone in man, *Ann. Int. Med.* **49**: 1090 (Nov.) 1958.
3. Medical Research Council Panel (England): The effectiveness in man of human growth hormone, *Lancet* **1**: 7 (Jan. 3) 1959.
4. Ikkos, D.; Luft, R., and Gemzell, C. A.: The effect of human growth hormone in man, *Acta endocrinol.* **32**: 341 (Nov.) 1959.
5. Wilhelmi, A. E.: Comparative biochemistry of growth hormone from ox, sheep, pig, horse and fish pituitaries, *in* The Hypophyseal Growth Hormone; Nature and Actions, ed. by R. Smith, D. Gaebler and C. N. H. Long. New York, Blakiston, 1954, p. 59.
6. Gemzell, C. A., and Heijenskjöld, F.: Growth hormone content in human pituitaries, *Endocrinology* **59**: 681 (Dec.) 1956.
7. Knobil, E.; Wolf, R. C., and Greep, R. O.: Some physiologic effects of primate pituitary growth hormone preparations in the hypophysectomized Rhesus monkey, (abstract) *J. Clin. Endocrinol. & Metab.* **16**: 916 (July) 1956.
8. Knobil, E.; Wolf, R. C.; Greep, R. O., and Wilhelmi, A. E.: Effects of a primate pituitary growth hormone preparation on nitrogen metabolism in the hypophysectomized Rhesus monkey, *Endocrinology* **60**: 166 (Jan.) 1957.

9. Li, C. H., and Papkoff, H.: Preparation and properties of growth hormone from human and monkey pituitary glands, *Science* **124:** 1293 (Dec.) 1956.
10. Li, C. H.; Papkoff, H.; Fonss-Bech, P., and Condliffe, P. G.: Action of chymotrypsin on hypophyseal growth hormone, *J. Biol. Chem.* **218:** 33 (Jan.) 1956.
11. Forsham, P. H.; Li, C. H.; DiRaimondo, V. C.; Kolb, F. O.; Mitchell, D., and Newman, S.: Nitrogen retention in man produced by chymotrypsin digests of bovine somatotropin, *Metabolism* **7:** 762 (Nov.) 1958.
12. Raben, M. S.: Preparation of growth hormone from pituitaries of man and monkey, *Science* **125:** 883 (May) 1957.
13. Lewis, U. J., and Brink, N. G.: Crystalline human growth hormone, *J. Am. Chem. Soc.* **80:** 4429 (Aug.) 1958.
14. Papkoff, H., and Li, C. H.: The isolation and characterization of growth hormone from sheep pituitary glands, *Biochem. & Biophys. acta* **29:** 145 (Jan.) 1958.
15. Papkoff, H., and Li, C. H.: The isolation and characterization of growth hormone from anterior lobes of whole pituitaries, *J. Biol. Chem.* **231:** 367 (Mar.) 1958.
16. Denko, C. W., and Bergenstal, D. M.: The effect of hypophysectomy and growth hormone on S^{35} fixation in cartilage, *Endocrinology* **57:** 76 (July) 1955.
17. Reifenstein, E. C., Jr.; Albright, F., and Wells, S. L.: The accumulation, interpretation, and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus, and nitrogen, *J. Clin. Endocrinol.* **5:** 367 (Nov.) 1945.
18. Bergenstal, D. M.; Lubs, H. A.; Hallman, L. F., and Schricker, J. A.: Effects of tolbutamide in the diabetes of acromegaly and on the blood sugar in patients with altered endocrine states, *Ann. New York Acad. Sc.* **71:** 215 (July) 1957.
19. Lipsett, M. B., and Bergenstal, D. M.: The failure of human growth hormone and ovine prolactin to stimulate human breast and prostate cancer, *Cancer Res.* (in press).
20. Bergenstal, D. M., and Lipsett, M. B.: The anabolic effect of sheep prolactin in man, *J. Clin. Invest.* **37:** 877 (June) 1958.
21. Evans, H. M.; Simpson, M. E., and Pencharz, R. I.: Relation between the growth promoting effects of the pituitary and the thyroid hormone, *Endocrinology* **25:** 175 (Aug.) 1939.
22. Batts, A. A.; Bennett, L. L.; Garcia, J., and Stein, J.: The effect of growth hormone on muscle potassium and on extracellular fluid, *Endocrinology* **55:** 456 (Oct.) 1954.
23. Ikkos, D.; Ljunggren, H., and Luft, R.: The relation between extracellular and intracellular water in acromegaly, *Acta endocrinol.* **21:** 212 (Mar.) 1956.
24. Becks, H.; Simpson, M. E.; Marx, W.; Li, C. H., and Evans, H. M.: Antagonism of pituitary adrenocorticotropic hormone (ACTH) to the action of growth hormone on the osseous system of hypophysectomized rats, *Endocrinology* **34:** 311 (May) 1944.

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RENAL CLEARANCE OF PITUITARY GONADOTROPINS IN POSTMENOPAUSAL WOMEN

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ABSTRACT

The renal clearance of human pituitary gonadotropins (HPG) was studied in 18 normal ambulant postmenopausal subjects and in 32 hospitalized postmenopausal patients suffering from various diseases. Individual values for clearance were low, ranging from 0.04 to 0.43 ml. per minute. When the mean renal clearance in the ambulant group was compared with that in the hospitalized group, no significant difference was found. For both urine and plasma the mean levels of HPG were significantly higher in the ambulant subjects than in the hospitalized patients.

THE renal clearance of human chorionic gonadotropin (HCG) in normal and abnormal pregnancy has already been extensively studied (1–3). Since the development of reasonably satisfactory methods for the quantitative determination of human pituitary gonadotropins (HPG) in urine (4) and in plasma (5), similar investigations with regard to these hormones are now possible. The purpose of the present paper is to report estimations of HPG clearance in normal postmenopausal subjects and in postmenopausal patients suffering from various diseases.

METHODS

Clinical studies

The renal clearance of HPG was investigated in a total of 50 postmenopausal women. Of these, 18 were normal ambulant subjects (Group I), 14 had recurrent and metastatic mammary carcinoma (Group II), and 18 were suffering from diseases other than cancer (Group III). The subjects in Groups II and III were hospitalized during the period of observation.

None of the women studied showed evidence of gross renal disease; in none of them was albuminuria present, or were there abnormal findings on microscopic examination of the urine. Creatinine clearances were performed in 24 of the 32 hospitalized patients; the technique described by Haugen and Blegen (6) was used. In only 4 subjects were the clearance values below 100 liters per twenty-four hours.

There were no endocrine abnormalities in any of the patients and in no case was hormonal therapy administered during the period of investigation.

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* M.R.C.P. (Ed.)

Methods of assay

Estimations of gonadotropin in plasma were performed by the method of Apostolakis (5) and in urine by the method of Loraine and Brown (4). For both plasma and urine, results of assays were expressed in terms of a reference preparation made from the urine of menopausal and postmenopausal subjects (HMG-20A); 1 "HMG unit" corresponds to the gonadotropic activity contained in 1 mg. of HMG-20A.

Calculation of renal clearance

This was determined by the formula UV/P, where U is the concentration of the hormone in the urine, P is the concentration in the plasma and V is the volume of urine excreted per minute. Clearance was expressed in milliliters per minute.

In addition to clearance, an estimation was made in each patient of the ratio of the gonadotropin concentration in plasma to that in urine (plasma/urine concentration ratio).

RESULTS

Renal clearance of HPG

a) *Normal ambulant subjects.* Estimations were made in 18 normal postmenopausal women. The results are shown in Table 1. The arithmetic mean of the 18 observations (ml. per minute) was 0.17 ± 0.01 (standard error of the mean). The fiducial range ($P = 0.95$) calculated logarithmically was $0.07 - 0.34$ ml. per minute. The mean plasma/urine concentration ratio was 6.4 (fiducial range, 3.2–11.2).

b) *Patients suffering from mammary carcinoma.* Fourteen patients were studied and 22 estimations were made (Table 2). During the entire period of observation the subjects in this group were hospitalized. The mean renal

TABLE 1. RENAL CLEARANCE AND PLASMA/URINE CONCENTRATION RATIOS OF HPG IN NORMAL POSTMENOPAUSAL WOMEN

Subject	Age (yrs.)	Urinary HPG (HMG units/ 24 hrs.)	Plasma HPG (HMG units/ 100 ml.)	Renal clearance (ml./min.)	Plasma/urine concentration ratio
J.H.	52	58	33	0.12	8.0
E.N.	58	46	14	0.23	3.1
J.S.	67	51	42	0.08	4.9
B.C.	52	72	36	0.14	3.3
A.M.	54	54	33	0.11	10.3
E.B.	53	71	34	0.15	6.8
C.L.	63	120	34	0.25	6.8
H.H.	56	47	17	0.19	5.2
C.M.	53	108	32	0.23	10.7
M.C.	59	83	28	0.21	4.9
M.O'B.	62	99	28	0.25	4.0
B.L.	56	60	24	0.17	5.0
A.M.	59	77	34	0.16	6.8
A.W.	52	117	36	0.23	5.5
A.B.	55	72	35	0.14	5.8
E.H.	61	90	47	0.13	10.4
S.F.	59	54	32	0.12	7.1
C.B.	77	78	38	0.14	5.8

TABLE 2. RENAL CLEARANCE AND PLASMA/URINE CONCENTRATION RATIOS OF HPG IN HOSPITALIZED PATIENTS WITH MAMMARY CARCINOMA

Patient	Age (yrs.)	Urinary HPG (HMG units/ 24 hrs.)	Plasma HPG (HMG units/ 100 ml.)	Renal clearance (ml./min.)	Plasma/urine concentration ratio
J.S.	58	31	14	0.15	8.4
		35	11	0.22	3.8
C.N.	53	31	15	0.14	10.6
E.Mc.K.	65	19	21	0.06	9.5
J.B.	49	60	16	0.26	2.0
		59	16	0.26	2.4
H.S.	86	19	26	0.05	26.0
		52	25	0.14	3.7
J.M.	44	136	22	0.43	3.8
G.W.	74	32	12	0.19	2.4
		52	15	0.24	2.6
C.K.	58	112	21	0.37	1.6
C.W.	66	47	12	0.27	4.4
C.M.	55	40	21	0.13	10.5
A.J.	73	59	34	0.12	3.0
		28	37	0.05	7.4
M.R.	55	54	24	0.16	4.8
		71	36	0.14	6.1
E.S.	59	84	38	0.18	7.4
		139	49	0.20	3.1
S.C.	61	62	20	0.22	4.8
		52	26	0.14	4.4

clearance (ml. per minute) was 0.19 ± 0.02 and the range ($P = 0.95$) was 0.05–0.50 ml. per minute. The mean plasma/urine concentration ratio was 6.0 and the fiducial range ($P = 0.95$) was 1.3–17.0.

c) *Patients suffering from diseases other than cancer.* Twenty-seven estimations were made in the 18 hospitalized patients in this group (Table 3). The mean renal clearance (ml. per minute) was 0.19 ± 0.02 and the fiducial range ($P = 0.95$) was 0.05–0.55. The mean plasma/urine concentration ratio was 3.9 and the fiducial range ($P = 0.95$) was 1.4–8.9.

Significance of results of renal clearance studies

The mean clearance values for HPG in the 3 groups of patients were very similar, being 0.17 ml. per minute in Group I and 0.19 ml. per minute in both Group II and Group III. Statistical analysis showed the absence of a significant difference between these mean values (Group I versus

TABLE 3. RENAL CLEARANCE AND PLASMA/URINE CONCENTRATION RATIOS OF HPG IN HOSPITALIZED PATIENTS SUFFERING FROM DISEASES OTHER THAN CANCER

Patient	Age (yrs.)	Diagnosis	Urinary HPG (HMG units/24 hrs.)	Plasma HPG (HMG units/100 ml.)	Renal clearance (ml./min.)	Plasma/urine concentration ratio
M.D.	67	Cholelithiasis	72	31	0.16	4.8
M.E.	73	Hypertension	20	10	0.14	4.2
W.McL.	57	Hypertension	65	20	0.23	2.4
			90	32	0.19	2.4
C.D.	50	Acromegaly	72	13	0.38	2.2
M.C.	58	Peptic ulcer	64	21	0.21	2.2
			72	12	0.41	1.3
J.F.	69	Cerebral arteriosclerosis	68	20	0.24	1.6
			51	34	0.10	4.3
J.B.	53	Rheumatoid arthritis	48	22	0.15	4.1
			70	27	0.18	4.2
W.H.	58	Hypertension	51	14	0.25	3.0
			120	20	0.41	2.5
E.McK.	52	Rheumatic heart disease	77	17	0.31	2.0
			22	16	0.10	5.0
E.O.	72	Diabetes mellitus	43	17	0.18	6.1
C.R.	63	Rheumatoid arthritis	20	17	0.08	7.1
R.D.	75	Enteritis	90	22	0.28	1.7
N.M.	72	Cardiac failure	9	18	0.04	8.6
			23	22	0.07	7.3
B.A.	60	Renal colic	86	25	0.24	3.7
E.A.	71	Hiatal hernia	61	55	0.08	4.0
C.B.	71	Cardiac failure; thyrotoxicosis	72	13	0.38	3.3
J.McL.	70	Epilepsy	45	47	0.07	5.9
			45	44	0.07	3.1
I.McL.	59	Peptic ulcer	42	34	0.09	3.4
			51	23	0.15	4.3

Group II, $P = 0.9–0.8$; Group I versus Group III, $P = 0.6–0.5$; Group I versus Groups II and III, $P > 0.9$).

Interdependence of urinary and plasma values for HPG

In view of the similarity of mean clearance values in all groups of patients, it was considered justifiable, when examining further the interde-

TABLE 4. HPG LEVELS IN URINE AND PLASMA IN AMBULANT AND HOSPITALIZED POSTMENOPAUSAL SUBJECTS

Group	No. of subjects	No. of observations	Mean urinary excretion (HMG units/24 hrs.)	S.D. (log)	Range (HMG units/24 hrs.) (P = 0.95)	Mean plasma concentration (HMG units/100 ml.)	S.D. (log)	Range (HMG units/100 ml.) (P = 0.95)
Normal ambulant subjects	18	18	76	0.17	35–158	32	0.14	17–59
Hospitalized patients with mammary carcinoma	14	22	55	0.31	14–224	21	0.17	10–46
Hospitalized patients with diseases other than cancer	18	27	51	0.26	16–166	22	0.17	10–47

pendence between gonadotropin levels in urine and plasma, to group together results obtained in all 50 subjects. The correlation coefficient of these two quantities was calculated and the value obtained was 0.33; this value is statistically significant ($P < 0.02$).

Urinary and plasma levels of HPG in ambulant and hospitalized subjects

These are presented in summary form in Table 4. Because of the wide variation in urinary excretion and plasma concentration in individual patients and between individuals, the results were calculated by the method described by Gaddum (7). Excretion values were converted into logarithms and the mean values, standard deviations and fiducial limits of error were then determined.

The data in Table 4 were subjected to an analysis of significance in which the mean urinary excretion and plasma concentration of HPG in the 18 ambulant subjects were compared with those in the 32 hospitalized patients. It was found that, both for urine and plasma, the mean gonadotropin levels were significantly higher in the ambulant group than in the hospitalized group ($P < 0.05$ for urine and < 0.01 for plasma).

DISCUSSION

The values for renal clearance reported in this study are calculated on the assumption that the yields of gonadotropins obtained by the method used for extraction of plasma are approximately equal to those obtained by the method used for the preparation of urinary extracts. It is obvious that if the yields from urine are consistently lower than the yields from plasma the calculated renal clearance will be erroneously low. If, on the other hand, the yields from urine are consistently higher than the yields from plasma the converse will be the case, and the mean value for clearance will be erroneously high. Present evidence indicates that the method of Apostolakis (5) for plasma and that of Loraine and Brown (4) for urine yield results which are of reasonable quantitative significance. For this

reason it would appear justifiable to use the values thus obtained for the calculation of renal clearance.

A further point which must be considered in the interpretation of results is whether the gonadotropins in plasma are qualitatively similar to those in urine. Although a direct comparison of plasma and urine extracts in the same patients has not yet been made, previous investigations (5, 8, 9) have shown that HMG-20A, the reference material used throughout this study, does not differ qualitatively from either type of extract and that therefore it is justifiable to express results of assays of both urine and plasma in terms of this material. It must be emphasised that the extracts

TABLE 5. RENAL CLEARANCE AND PLASMA/URINE CONCENTRATION RATIOS FOR HPG AND HCG

Hormone	Source	No. of subjects	No. of observations	Renal clearance (ml./min.)		Plasma/urine concentration ratio		Reference
				Mean value	Arithmetic range	Mean value	Arithmetic range	
HPG	Postmenopausal women	50	67	0.18±0.02*	0.04–0.43	5.3	1.3–26.0	This paper
HCG	Normally pregnant women	12	32	0.95±0.03	0.69–1.19	1.1	0.6–2.5	Loraine (3)
HCG	Normally pregnant women	4	20	0.38±0.03	0.24–0.79	3.1	1.0–5.0	Gastineau, Albert & Randall (1)

* Standard error of the mean.

used in this study were prepared by the kaolin-acetone method for urine and by the acetone precipitation-dialysis procedure for plasma. It is probable that, had other methods of extraction been employed, the materials obtained would have been qualitatively dissimilar. For further information on the criteria of dissimilarity, articles by Gaddum (10) and by Borth and associates (11) should be consulted.

The low values for the renal clearance of HPG in this study are typical of those reported for other proteins and it would appear reasonable to assume that the total amount of HPG finally excreted in the urine represents a small fraction only of the quantity presented to the kidney. The mean renal clearance in all three groups of patients was approximately the same and this fact indicates that renal clearance is not affected by the ambulatory or non-ambulatory status of the subject or by the presence of various diseases not associated with renal failure. It should be emphasised that, although renal clearance values for HPG remained relatively constant in all three groups of patients, values both for urinary excretion and plasma concentration were significantly higher in the normal ambulant postmenopausal subjects than in the hospitalized postmenopausal patients suffering from various diseases (Table 4). This finding will be considered in more detail in a subsequent publication (12).

A comparison of the results obtained in this study with the data reported by other investigators for renal clearance and plasma/urine concentrations of HCG is shown in Table 5. The relatively wide difference in clearance figures and plasma/urine concentration ratios for HCG reported by Loraine (3) on the one hand and by Gastineau and associates (1) on the other should be noted. The reasons for this discrepancy are probably of methodologic origin and have been discussed elsewhere (3, 13, 14). It is, however, apparent that in all three studies the renal clearance of HCG was considerably higher than that of HPG and that the converse was true for plasma/urine concentration ratios.

The fact that a marked difference exists between the renal clearances of pituitary and placental gonadotropins is not of itself surprising in view of the many chemical and biologic dissimilarities between the two groups of hormones. At the time of writing, the exact mechanism by which gonadotropins are "cleared" by the kidney is not known and therefore no definite theory can be advanced to explain the observed difference. This question may well be a rewarding subject for future study.

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REFERENCES

1. Gastineau, C. F.; Albert, A., and Randall, L. M.: The renal clearance of chorionic gonadotropic hormone in pregnancy, in neoplasm of the testis and in hydatidiform mole, *J. Clin. Endocrinol.* **9**: 615, 1949.
2. Johnson, C. E.; Albert, A., and Wilson, R. B.: Renal and extrarenal disposal of chorionic gonadotropin in immediate postpartum period, *J. Clin. Endocrinol.* **10**: 371, 1950.
3. Loraine, J. A.: The renal clearance of chorionic gonadotrophin in normal and pathological pregnancy, *Quart. J. Exper. Physiol.* **36**: 11, 1951.
4. Loraine, J. A., and Brown, J. B.: A method for the quantitative determination of gonadotrophins in the urine of non-pregnant human subjects, *J. Endocrinol.* (London) **18**: 77, 1959.
5. Apostolakis, M.: Detection and estimation of pituitary gonadotrophins in human plasma, *J. Endocrinol.* (London) **19**: 377, 1959.
6. Haugen, H. N., and Blegen, E. M. The true endogenous creatinine clearance, *Scandinav. J. Clin. & Lab. Invest.* **5**: 67, 1953.
7. Gaddum, J. H.: Lognormal distributions, *Nature* (London) **156**: 463, 1945.
8. Apostolakis, M., and Loraine, J. A.: Pituitary gonadotrophins in blood, *in* Hormones in Blood, ed. by C. H. Gray and A. L. Bacharach. New York, Academic Press, Inc., 1960 (in press).

9. Loraine, J. A., and Brown, J. B.: Further observations on the estimation of urinary gonadotropins in non-pregnant human subjects, *J. Clin. Endocrinol. & Metab.* **16:** 1180, 1956.
10. Gaddum, J. H.: Polypeptides Which Stimulate Plain Muscle. Edinburgh, Livingstone, 1955, p. 133.
11. Borth, R.; Linder, A., and Lunenfeld, B.: Relative potency of human pituitary preparations from urine in different types of bioassay, *Acta endocrinol.* **31:** 192, 1959.
12. Apostolakis, M., and Loraine, J. A.: In preparation, 1960.
13. Diczfalusy, E., and Loraine, J. A.: Sources of error in clinical bioassays of serum chorionic gonadotropin, *J. Clin. Endocrinol. & Metab.* **15:** 424, 1955.
14. Loraine, J. A.: The Clinical Application of Hormone Assay. Edinburgh, Livingstone, 1958, p. 91.

RENAL CLEARANCE OF ENDOGENOUS 17-HYDROXYCORTICOSTEROIDS IN HYPERTENSION*

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ABSTRACT

Renal clearances of endogenous free and conjugated 17-hydroxycorticosteroids (17-OH-CS) were determined in 15 normotensive and 13 hypertensive subjects (12 with essential hypertension and one with Cushing's syndrome) and were related to endogenous creatinine clearance. Free 17-OH-CS were cleared at similar rates in the two groups of subjects. These rates were 15–25 times lower than those for conjugated 17-OH-CS. After stimulation with ACTH the clearance of free 17-OH-CS increased significantly in both groups as compared with the clearance under basal conditions. The clearance of total conjugated 17-OH-CS (extractable with butanol-chloroform mixture) *was significantly lower in the hypertensive than in the normotensive subjects.* In patients with renal damage the percentage decrease in the clearance of total conjugated 17-OH-CS exceeded the decrease in creatinine clearance. The decreased clearance of total conjugated 17-OH-CS resulted in the accumulation of these steroids in the blood, thus decreasing the ratio of free to conjugated 17-OH-CS in plasma. The 17-OH-CS glucuronides (released by β-glucuronidase hydrolysis) were cleared at similar rates in normotensive and hypertensive subjects. In patients with decreased renal function these rates were proportional to those of creatinine clearance. Moreover, the concentration of 17-OH-CS glucuronides in the urine of hypertensive patients was almost identical with the concentration of total conjugated 17-OH-CS in the same specimens. After administration of ACTH, the clearance of total conjugated 17-OH-CS decreased in normotensive subjects but increased slightly in hypertensive patients, as compared with the corresponding clearances under basal conditions. Thus, the hypertensive patients under basal conditions cleared total conjugated 17-OH-CS in a manner similar to that observed in the normotensive subjects after administration of ACTH.

DURING recent years evidence has accumulated that the adrenal cortex plays an important role in the maintenance of arteriolar tone and is essential for the development and maintenance of arterial hypertension (2–6). However, the nature of the relationship of adrenocortical

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secretion to high blood pressure has not been elucidated. Several workers investigated the possibility of increased secretion of adrenocortical steroids in arterial hypertension, but no significant changes were found (7–13). Recently Genest *et al.* (14) reported that the mean urinary excretion of aldosterone in 39 hypertensive patients was about twice the average excretion in normal subjects. However, the fluctuations were considerable, and in most of the hypertensive subjects the values for aldosterone excretion were within the upper limit of the normal range.

A survey of the methods used in these investigations reveals that, in general, only the "free" fraction of the corticoids was estimated in blood, whereas the "total" (free and conjugated) corticoids were measured in urine. Furthermore, methods used in most of these studies were of low sensitivity and unsuitable for determination of steroids in blood.

The object of the present work was to reinvestigate the relationship between the adrenal cortex, kidney and high blood pressure, by determining the renal clearance of endogenous free and conjugated 17-hydroxycorticosteroids (17-OH-CS) and relating it to renal function measured simultaneously by a creatinine clearance test.

METHODS AND MATERIAL

Free and conjugated 17-OH-CS in plasma and urine were determined by methods previously published (15–17).

Renal clearance of free and conjugated 17-OH-CS was calculated from the concentrations of these steroids in urinary specimens collected over the one-hour period between 9:30 and 10:30 a.m. and in plasma drawn at 10 a.m.[1] In a preliminary investigation it was found that the most reproducible results were obtained when the test was carried out over a one-hour period. Since it was noticed that increased diuresis was associated with increased urinary excretion of 17-OH-CS, particularly of the free steroids, the tests were performed under conditions of a moderately declining diuresis, the patients drinking 200 ml. of water fifteen minutes before the start of the collection of urine.

Endogenous creatinine clearance was determined simultaneously with that of 17-OH-CS; urinary and serum creatinine levels were estimated by the method of Løken (18).

Fifteen normal subjects and thirteen hypertensive patients were investigated.

The group of *normal subjects* consisted of 12 men ranging in age from 24 to 53 years, and 3 women aged 28, 32 and 44. Blood pressure in this group ranged between 105/60 and 145/90 mm. Hg, the mean being 132/78.

The *hypertensive group* consisted of 12 patients with essential hypertension—10 men ranging in age from 25 to 56 years, and 2 women aged 49 and 58—and 1 patient with Cushing's syndrome, a women aged 27. Blood pressure in this group ranged between 170/100 and 240/160 mm. Hg, the mean being 199/117.

The renal clearance of 17-OH-CS was also determined after stimulation with adreno-

[1] The early morning hours were found to be the most convenient for the test, as at that time of day the blood levels of 17-OH-CS were high enough to assure the desirable precision of the estimation.

corticotropic hormone (ACTH). The ACTH test was performed in 6 normal subjects—4 men (age range, 24–37) and 2 women (ages 28 and 32)—and in 8 patients with essential hypertension—7 men (age range, 38–51) and 1 woman (age 58); the test was also performed in the patient with Cushing's syndrome. In these subjects a dose of 120 I.U. of ACTH-gel was administered at midnight in one intramuscular injection. The following morning the one-hour renal clearance of endogenous free and conjugated 17-OH-CS between 9:30 and 10:30 a.m. was determined. A preliminary investigation revealed that six to eight hours after one intramuscular injection of ACTH-gel, a plateau occurs in the levels of free and conjugated plasma 17-OH-CS, which is still maintained twelve hours after the injection (19).

In the following text the clearance of 17-OH-CS under normal resting conditions will be referred to as "basal clearance"; the 17-OH-CS clearance during administration of ACTH will be referred to as "clearance with ACTH."

In 7 normal subjects and 5 patients with essential hypertension the renal clearance of 17-OH-CS released by β-glucuronidase hydrolysis was determined simultaneously with that of the butanol-chloroform extractable conjugated 17-OH-CS, and the results were compared. In the following text the 17-OH-CS released by β-glucuronidase hydrolysis will be referred to as "17-OH-CS-glucuronides" and the butanol-chloroform extractable steroids as "total conjugated 17-OH-CS."

In all subjects the blood pressure was recorded immediately after withdrawal of blood. Repeated blood pressure measurements were also made before the performance of the clearance test at intervals of a few days. All the blood pressure readings were obtained with a mercury sphygmomanometer by the auscultatory technique, the point of the complete disappearance of the sound being considered as corresponding to the diastolic pressure.

RESULTS

Plasma levels and urinary excretion of free and conjugated 17-OH-CS

From Table 1 it may be seen that although no difference was found between the plasma levels of free 17-OH-CS in the two groups of subjects, the levels of conjugated 17-OH-CS were significantly higher in the hypertensive than in normotensive subjects ($P < 0.01$). It is of interest that in both groups there was no substantial difference between the plasma levels of total conjugated 17-OH-CS and those of 17-OH-CS glucuronides (Table 2).

TABLE 1. PLASMA LEVELS AND URINARY EXCRETION OF FREE AND CONJUGATED 17-OH-CS

Subjects	Conditions of study	No. of subjects	Plasma levels of 17-OH-CS (μg./100 ml.)		Sixty-minute urinary excretion of 17-OH-CS (μg.)	
			Free	Total conjugated	Free	Total conjugated
Normotensive	Basal	15	13.4±2.8* (7.5–27.0)†	14.1±2.7 (6.0–28.0)	32.0±5.6 (18.0–55.0)	870.0±85.0 (504.0–1260.0)
Hypertensive	Basal	12	13.6±2.6 (6.5–23.0)	27.4±4.2 (15.0–51.0)	43.0±8.7 (25.9–90.0)	886.0±90.0 (334.0–1365.0)
Normotensive	ACTH	6	45.3±3.1 (33.0–51.4)	66.8±10.2 (36.0–100.0)	248.0±27.0 (170.0–324.0)	3248.0±460.0 (1848.0–4586.0)
Hypertensive	ACTH	9	50.9±5.0 (30.0–62.0)	77.7±12.0 (52.0–135.0)	355.0±58.0 (185.0–702.0)	3723.0±820.0 (1428.0–8384.0)

* Mean and standard deviation.
† Range.

TABLE 2. PLASMA AND URINARY CONCENTRATIONS OF 17-OH-CS-GLUCURONIDES AND TOTAL CONJUGATED 17-OH-CS UNDER BASAL CONDITIONS

Subjects	No. of subjects	No. of estimations	Plasma 17-OH-CS (μg./100 ml.)		Urinary 17-OH-CS (μg./100 ml.)		
			Gluc. conjug.	Tot. conjug.	Gluc. conjug.	Tot. conjug.	Gluc. conjug. as % of total conjug.
Normotensive	7	12					
Range			8–28	9–29	245–3040	360–4500	60–85
Mean			14.8	15.6	906	1240	73%
Hypertensive	5	10					
Range			14.5–52	15–51	230–2500	220–2550	96–104
Mean			26.0	27.0	980	980	100%

After the administration of ACTH the differences between the levels of conjugated 17-OH-CS in normal and hypertensive subjects decreased considerably as compared to the corresponding differences under basal conditions (Table 1).

The urinary excretion of free 17-OH-CS over a sixty-minute period was slightly but significantly higher in the hypertensive than in the normotensive subjects ($P < 0.01$), both under basal conditions and after administration of ACTH. The excretion of total conjugated 17-OH-CS was roughly the same in the two groups of subjects under basal conditions and with ACTH. However, the range of the excretion of these conjugates after administration of ACTH was considerably wider in the hypertensive than in the normotensive subjects (Table 1).

In the urinary specimens of the normotensive subjects under basal conditions the 17-OH-CS glucuronides constituted about 75 per cent of the total conjugated 17-OH-CS. There was, however, no conspicuous difference between the concentrations of 17-OH-CS glucuronides and of total conjugated 17-OH-CS in the urinary specimens of hypertensive patients (Table 2).

The basal renal clearance of free and conjugated 17-OH-CS

From Table 3 it is evident that there was no substantial difference between the renal clearances of free 17-OH-CS in normotensive and hypertensive subjects. However, the renal clearance of total conjugated 17-OH-CS was significantly lower in the hypertensive than in the normotensive subjects ($P < 0.001$). The decrease in the clearance of these conjugates occurred even in hypertensive patients with normal creatinine clearance. In hypertensive patients with renal damage the decrease in the clearance

TABLE 3. BASAL RENAL CLEARANCE OF FREE AND CONJUGATED 17-OH-CS

Subjects	Creatinine clearance, plasma (ml./min.)	Renal clearance of free 17-OH-CS		Renal clearance of total conjugated 17-OH-CS	
		plasma (ml./min.)	% of creat. clearance	plasma (ml./min.)	% of creat. clearance
Normotensive					
Range	76–155	2.7–9.2	2.1–10.0	90–189	66–140
Mean	130	4.7	3.8	124	98
Hypertensive					
Essential hypertension					
Range	40–198	1.9–9.6	2.0–6.9	31–90	32–79
Mean	119	5.7	5.1	57	51
Cushing's syndrome	76	4.3	5.6	50	65

of total conjugated 17-OH-CS exceeded the decrease in creatinine clearance. In the patient with Cushing's syndrome the clearance of both free and conjugated 17-OH-CS fell within the clearance range observed in patients with essential hypertension.

The 17-OH-CS glucuronides were cleared at similar rates in both groups. These rates were considerably lower than those of the simultaneously determined clearances of total conjugated 17-OH-CS in normal subjects, but they were almost identical with the rates of clearance of total conjugated 17-OH-CS in hypertensive patients (Fig. 1).

The renal clearance of free and conjugated 17-OH-CS after administration of ACTH

From Table 4 it can be seen that after administration of ACTH the free 17-OH-CS were cleared at slightly higher rates in hypertensive than in normotensive subjects. This difference was statistically significant (P

TABLE 4. RENAL CLEARANCE OF FREE AND CONJUGATED 17-OH-CS AFTER ADMINISTRATION OF ACTH

Subjects	Creatinine clearance, plasma (ml./min.)	Free 17-OH-CS		Total conjugated 17-OH-CS	
		plasma (ml./min.)	% of creat. clearance	plasma (ml./min.)	% of creat. clearance
Normotensive					
Range	120–163	4.8–16.2	4.0–11.7	61–104	40–75
Mean	147	9.4	6.4	83	56
Hypertensive					
Essential hypertension					
Range	87–260	5.8–18.9	6.7–10.7	46–162	42–68
Mean	149	12.7	8.8	84	56
Cushing's syndrome	64	4.1	6.4	46	70

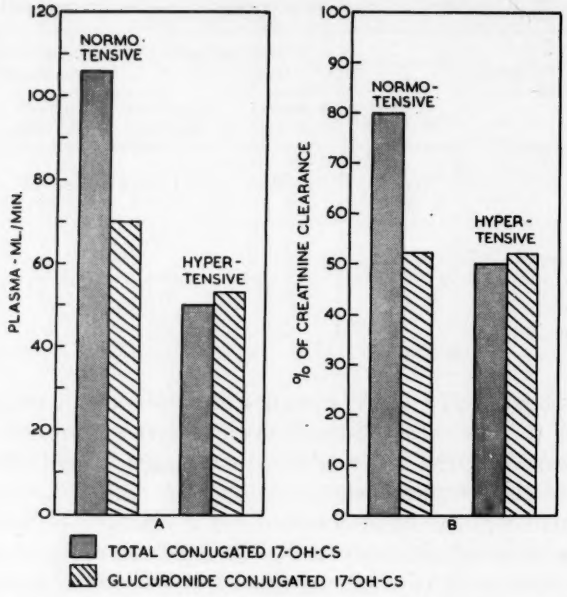

FIG. 1. Mean values for the basal renal clearance of total conjugated 17-OH-CS and 17-OH-CS glucuronides. **A.** Absolute values in ml. of plasma per minute. **B.** Values related to creatinine clearance.

<0.01). In both groups there was a significant increase in the clearance rates of free 17-OH-CS after administration of ACTH as compared with the clearance under basal conditions (Table 5).

In normotensive subjects after injection of ACTH the total conjugated 17-OH-CS were cleared at rates significantly lower than under basal conditions ($P < 0.001$). In contrast, the hypertensive patients cleared total conjugated 17-OH-CS at similar rates, both under basal conditions and following ACTH (Table 5). This resulted in an almost complete reduction of the differences between the clearance rates of total conjugated 17-OH-CS in normotensive and hypertensive subjects after administration of ACTH.

Ratio of free to conjugated 17-OH-CS in blood

The decreased clearance of conjugated 17-OH-CS in hypertensive patients led to elevation of the plasma levels of these steroids, thus altering the ratio of free to conjugated 17-OH-CS in blood. In the hypertensive group, the mean ratio was 1:2.2 (range, 1:1.6–1:4.3), whereas in the normotensive group the ratio was 1:1.1 (range, 1:0.4–1:1.8). The difference between these means was statistically significant ($P < 0.01$).

After administration of ACTH the ratio of free to conjugated 17-OH-CS

TABLE 5. MEAN VALUES FOR RENAL CLEARANCE OF FREE AND CONJUGATED 17-OH-CS UNDER BASAL CONDITIONS AND AFTER STIMULATION WITH ACTH*

	Creatinine clearance (ml./min.)	Free 17-OH-CS		Total conjugated 17-OH-CS	
		ml./min.	% creat. clearance	ml./min.	% creat. clearance
Normotensive subjects					
Mean "basal"	130	3.9	3.1	108	82
Mean after ACTH	147	9.4	6.4	83	56
Mean increase	17 (14%)	5.5 (141%)	3.3 (106%)	—	—
Mean decrease	—	—	—	25 (23%)	26 (32%)
Hypertensive subjects					
Mean "basal"	122	5.4	4.6	61	54
Mean after ACTH	139	11.7	8.5	80	57
Mean increase	17 (14%)	6.3 (117%)	3.9 (85%)	19 (31%)	3 (6%)
Mean decrease	—	—	—	—	—

* Calculated from the results obtained in the group of patients who were investigated under both basal conditions and after administration of ACTH.

in blood did not differ substantially between the two groups of subjects. In the normotensive subjects it was 1:1.4 (range, 1:1.0–1:1.9); in the hypertensive patients it was 1:1.6 (range, 1:1.0–1:2.7).

Ratio of free to conjugated 17-OH-CS in urine

The mean ratio of free to conjugated 17-OH-CS in urine was 1:28 (range, 1:13–1:40) in the normotensive subjects, and 1:23 (range, 1:10–1:40) in the hypertensive patients.

After injection of ACTH, the mean ratio of free to conjugated 17-OH-CS in urine was 1:14 (range, 1:7–1:27) in normotensive subjects, and 1:11 (range, 1:7–1:18) in hypertensive patients.

Thus there was a considerable increase in the ratio of free to conjugated 17-OH-CS in urine in both normal and hypertensive subjects after administration of ACTH as compared to the ratio under "basal" conditions.

DISCUSSION

The data presented in this work indicate that the conjugated 17-OH-CS extractable with a butanol-chloroform mixture (total conjugated 17-OH-CS) are cleared at significantly lower rates in hypertensive patients than in normotensive subjects. This results in accumulation of these con-

jugates in the blood, with a consequent alteration in the blood ratio of free to conjugated 17-OH-CS.

The ability to demonstrate the presence of conjugated 17-OH-CS not hydrolyzable by β-glucuronidase in the urine of normotensive subjects is contrasted by the inability to demonstrate such conjugates, at least in measurable concentrations, in the plasma. It appears that methodologic reasons for this "discrepancy" can be ruled out, as the extraction procedure used for both plasma and urine was essentially the same, and no Porter-Silber chromogens were lost during the deproteinization and purification procedures for plasma prior to the extraction (16). Furthermore, under the experimental conditions employed, a complete enzymatic hydrolysis of pure tetrahydrocortisone monoglucuronide was obtained (17). It therefore seems justified to assume that the conjugated 17-OH-CS not hydrolyzable by β-glucuronidase (or at least a major portion of these steroids) are cleared by the kidney so rapidly that they do not attain measurable concentrations in the plasma. The steroidal nature of the non-enzyme-hydrolyzable increment in the total conjugated 17-OH-CS is indicated by the presence of C^{14} in this fraction after intravenous administration of tracer doses of cortisol 4-C^{14} (20). In addition, this increment shows a distinct peak at 410 mμ with the Porter-Silber reagent and a positive reaction with blue tetrazolium. Furthermore, a part of these conjugates (25–30 per cent) can be cleaved by mild acid hydrolysis at 37° C., yielding tetrahydrocortisol, as demonstrated by paper chromatographic separation (21). A more detailed investigation into the nature of these conjugates is under way.

The 17-OH-CS released by β-glucuronidase hydrolysis (17-OH-CS glucuronides) are cleared at similar rates in normotensive and hypertensive subjects. In the normotensive group these rates are considerably lower than the rates of clearance of the total conjugated 17-OH-CS, whereas in the hypertensive group the rates are almost identical. Since the rate of clearance of total conjugated 17-OH-CS is a sum of clearance rates of 17-OH-CS glucuronides and of conjugated 17-OH-CS not hydrolyzable by β-glucuronidase, it can be adduced that the latter conjugates are not cleared by the kidney in patients with essential hypertension. Two possibilities suggest themselves: a) they may be retained in the circulation due to formation of a complex with another substance (more firmly bound with proteins or polypeptides) which is too big to be cleared by the kidney, or b) they are not synthesized, or synthesized at a much lower rate, in patients with essential hypertension. If the first possibility were true, the plasma concentrations of the conjugated 17-OH-CS not hydrolyzable by β-glucuronidase, but extractable with butanol-chloroform mixture, should be increased in hypertensive patients. This, however, could not be demonstrated in the present study. If it be true that patients with essential hy-

pertension do not possess, or possess only a reduced ability to conjugate 17-OH-CS in other forms than glucuronides, almost all of the 17-OH-CS secreted by the adrenal cortex in these patients would have to be conjugated with glucuronic acid. This could mean an increased load upon the mechanism of conjugation with glucuronic acid, which, when persisting for a long time, might result in an alteration of the pattern of conjugation.

The participation of the unsaturated ketone at C3 of the steroid nucleus in the binding of free corticoids with proteins has been suggested by Westphal (22). The reduction of this unsaturated ketone in 17-OH-CS glucuronides has been assumed to be one of the reasons for the decreased binding of these conjugates with plasma proteins (23, 24). It is possible that under the assumed changed conditions of corticosteroid conjugation

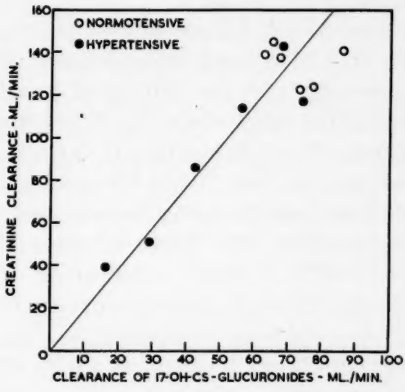

FIG. 2. Correlation between the renal clearance of 17-OH-CS glucuronides and creatinine clearance.

in patients with essential hypertension, a part of the 17-OH-CS is conjugated with glucuronic acid before being converted into tetrahydrometabolites. Persistence of the unsaturated ketone at C3 in such conjugates would result in their increased or more firm binding with the plasma proteins (or polypeptides).

During stimulation with ACTH the renal clearance of total conjugated 17-OH-CS decreases in normotensive subjects and increases slightly in hypertensive subjects; consequently, the differences detected between the two groups under basal conditions almost completely disappear after administration of ACTH. Moreover, hypertensive patients under basal conditions clear total conjugated 17-OH-CS in a manner similar to that of the normotensive subjects receiving ACTH. These findings suggest the possibility of a change in the pattern of conjugation in normotensive subjects during stimulation with ACTH.

From a comparison (Fig. 2) of the rates of clearance of 17-OH-CS

glucuronides with the rates of the corresponding creatinine clearance in hypertensive and normotensive subjects, it can be seen that the clearance of 17-OH-CS glucuronides decreases proportionately with the decrease in creatinine clearance. It can also be seen that the rates of excretion of the total conjugated 17-OH-CS correlate roughly with the rates of creatinine clearance. This is compatible with the findings of Englert *et al.* (25) who reported increased plasma levels of conjugated 17-OH-CS in patients with uremia. It is therefore concluded that the clearance of conjugated 17-OH-CS is decreased in the presence of impairment in renal function. Consequently, patients with so-called renal hypertension will also exhibit increased levels of plasma conjugated 17-OH-CS as a result of decreased clearance by the damaged kidneys. This concept, subject to additional confirmation, suggests a common link in the mechanisms of essential and renal hypertension.

Free 17-OH-CS are cleared at considerably lower rates than conjugated 17-OH-CS in both normotensive and hypertensive subjects. This is in agreement with the findings of Bongiovanni and Eberlein (26) who determined the renal clearance of free and conjugated 17-OH-CS in 1 subject after oral administration of large doses of tetrahydrocortisone. The low clearance of free 17-OH-CS can be explained by the very high percentage of these steroids being bound to plasma protein (23, 27) and by tubular reabsorption (19). After administration of ACTH the clearance of free 17-OH-CS increases significantly. This is presumably due to the high plasma levels of these steroids, which considerably exceed the cortisol-binding capacity of transcortin, a plasma protein described by Slaunwhite and Sandberg (27) that has a high affinity for cortisol but is present in low concentrations.

Apart from all the considerations regarding conjugation and clearance of 17-OH-CS, the possibility of a mild adrenal hyperfunction in essential hypertension is suggested by the interrelation of the values expressing the plasma levels and the urinary excretion of 17-OH-CS. The levels of free 17-OH-CS in plasma are similar in normotensive and hypertensive subjects, whereas the sixty-minute urinary excretion is slightly higher in hypertensive patients; the levels of 17-OH-CS conjugates in the plasma of hypertensive patients are considerably higher than in normotensive subjects, but the sixty-minute urinary excretion is roughly the same in both groups. This points to an increased production of both free and conjugated 17-OH-CS in patients with essential hypertension. However, in view of the fact that urinary corticoid excretion was estimated only during a small fraction of the day (sixty minutes), these observations may only indicate hyperactivity of the adrenals during the morning hours in hypertensive patients.

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REFERENCES

1. Kornel, L.: Renal clearance of free and conjugated 17-hydroxycorticosteroids in normotensive and hypertensive subjects. Preliminary communication, *Lancet* **2**: 775, 1957.
2. Perera, G. A.: Relationship of adrenal cortex to hypertension; observations on effect of hypoadrenalism on patient with hypertensive vascular disease, *J.A.M.A.* **129**: 537, 1945.
3. Arnott, W. M.; Crooke, A. C.; Donovan, H., and Taylor, S. H.: Total adrenalectomy for severe hypertension, *Quart. J. Med.* **26**: 501, 1957.
4. Goldblatt, H.: Studies on experimental hypertension: pathogenesis of experimental hypertension due to renal ischemia, *Ann. Int. Med.* **11**: 69, 1937.
5. Floyer, M. A.: The effect of nephrectomy and adrenalectomy upon the blood pressure in hypertensive and normotensive rats, *Clin. Sc.* **10**: 405, 1951.
6. Kornel, L.: Kidney, adrenal cortex and hypertension. Theory and facts, *A.M.A. Arch. Int. Med.* **103**: 820, 1959.
7. Bruger, M.; Rosenkranz, J. A., and Lowenstein, B. E.: Studies on morphology of adrenal cortex and on excretion of 17-ketosteroids in hypertensive patients, *Am. J. M. Sc.* **208**: 212, 1944.
8. Selye, F. L.: Biochemical changes in hypertension, *Canad. M. A. J.* **57**: 325, 1947.
9. Daughaday, W. H.; Jaffe, H., and Williams, R. H.: Adrenal cortical hormone excretion in endocrine and nonendocrine disease as measured by chemical assay, *J. Clin. Endocrinol.* **8**: 244, 1948.
10. Tobian, L., Jr., and Joseph, H.: Adrenal cortex activity in essential hypertension, (abstract) *J. Clin. Endocrinol.* **9**: 677, 1949.
11. Corcoran, A. C.; Page, I. H., and Dustan, A. P.: Urinary formaldehydogenic corticoids; normal values and observations in hypertension, *J. Lab. & Clin. Med.* **36**: 297, 1950.
12. Hetzel, B. S., and Hine, D. C.: Adrenal cortical function in hypertension, *J. Clin. Endocrinol.* **12**: 296, 1952.
13. Genest, J.: The relationship between sodium, arterial hypertension and the adrenal gland, *in* Ciba Foundation Symposium on Hypertension, ed. by G. Wolstenholme and M. Cameron, London, J. & A. Churchill, Ltd., 1954, p. 203.
14. Genest, J.; Koiw, E.; Nowaczynski, W., and Leboeuf, G.: Further studies on urinary aldosterone in human arterial hypertension, *Proc. Soc. Exper. Biol. & Med.* **97**: 676, 1958.
15. Kornel, L.: An improved rapid method for free and conjugated 17-hydroxycorticosteroids in urine, *Metabolism* **8**: 432, 1959.

16. KORNEL, L.: An improved rapid method for free and conjugated 17-hydroxycorticosteroids in plasma, *J. Lab. & Clin. Med.* **54:** 659, 1959.
17. KORNEL, L.: A modification of the Silber-Porter method for urinary 17-hydroxycorticosteroids conjugated with glucuronic acid, *Metabolism* **9:** 82, 1960.
18. LØKEN, F.: On the determination of creatinine in plasma by the Jaffé reaction, after absorption to Lloyd's reagent, *Scandinav. J. Clin. & Lab. Invest.* **6:** 325, 1954.
19. SCHEDL, H. P.; CHEN, P. S.; GREENE, G., and REDD, D.: The renal clearance of plasma cortisol, *J. Clin. Endocrinol. & Metab.* **19:** 1223, 1959.
20. EIK-NES, K.: Personal communication.
21. KORNEL, L., and HILL, S. R., JR.: Paper chromatographic pattern of endogenous urinary corticosteroids in normal subjects, *Metabolism* (in press).
22. WESTPHAL, U.: Spectrophotometric demonstration of interaction between proteins and delta^4-3-ketosteroid hormones, *Fed. Proc.* **15:** 382, 1956.
23. DAUGHADAY, W. H.: Binding of corticosteroids by plasma proteins. I. Dialysis equilibrium and renal clearance studies, *J. Clin. Invest.* **35:** 1428, 1956.
24. DAUGHADAY, W. H.: Binding of corticosteroids by plasma proteins. II. Paper electrophoresis and equilibrium paper electrophoresis, *J. Clin. Invest.* **35:** 1434, 1956.
25. ENGLERT, E.; BROWN, H.; WILLARDSON, D. G.; WALLACH, S., and SIMONS, E. L.: Metabolism of free and conjugated 17-hydroxycorticosteroids in subjects with uremia, *J. Clin. Endocrinol. & Metab.* **18:** 36, 1958.
26. BONGIOVANNI, A. M., and EBERLEIN, W. R.: Determination, recovery, identification and renal clearance of conjugated adrenal corticoids in human peripheral blood, *Proc. Soc. Exper. Biol. & Med.* **89:** 281, 1955.
27. SLAUNWHITE, W. R., and SANDBERG, A. A.: Transcortin: a cortiocosteroid-binding protein of plasma, *J. Clin. Invest.* **38:** 384, 1959.

HYPERURICEMIA IN MYXEDEMA*

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ABSTRACT

Serum uric acid levels were determined in 28 myxedematous patients and compared to the levels in 7 hyperthyroid patients and 33 normal subjects. Urinary urate excretions were also measured in 18 patients and 24-hour uric acid excretion rates determined in 16. The data indicate that most of the myxedematous males and one-third of the myxedematous females had significant hyperuricemia. Postmenopausal women were the only females in the myxedematous group exhibiting hyperuricemia. Myxedematous patients of both sexes showed a decreased urinary excretion of urate and a decreased renal urate excretion rate as compared to hyperthyroid patients. Amounts of triiodothyronine or triiodothyropropionic acid which were sufficient to elevate the basal metabolic rate to normal in the myxedematous patients studied, caused a urate diuresis, an increase in the 24-hour urate excretion rate, and a decrease in the level of serum uric acid.

HYPOTHYROIDISM in the human has been associated with a variety of abnormalities in the blood and urine. In the process of another investigation, we noted that certain patients with myxedema exhibit hyperuricemia. Since this observation had not been previously reported, we studied the frequency of this occurrence in a group of patients with myxedema, and also the relationship of thyroid function to the 24-hour renal excretion rate and total excretion of urates.

METHODS AND MATERIALS

Serum uric acid concentration was determined in the Clinical Biochemistry Laboratory of Memorial Center, by a modification of Archibald's (1) adaptation of the

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Kern-Stransky method (2). The 24-hour urinary excretion of urate was determined by the same method after treatment of an aliquot of the total urine sample with uricase. Excretion rates were calculated from the 24-hour urinary excretion and the serum uric acid level for the same day. All serum values represent the average of two or more determinations, and values for urate excretion represent averages for three or more consecutive specimens. Excretion rates are averages of two or more determinations, not necessarily consecutive. Blood urea nitrogen concentration was determined in most patients.

Diets, when employed as part of the study, were of three types. The low purine diet (L) contained little meat, with a daily intake of less than 300 mg. of purine; the moderate purine diet (M) contained 300–500 mg. of purine; and the regular hospital diet (R) averaged 500–700 mg. of purine.

Serum uric acid levels were determined in 28 patients in whom myxedema had been definitely diagnosed on the basis of clinical and laboratory evidence. Twelve of these patients were males and 16 were females. Simultaneously, serum was collected from 33 normal hospital employees who served as controls; both serum uric acid and blood urea nitrogen (BUN) determinations were carried out on these samples. There was no family history of gout or renal disease in the control group.

Urinary urate excretion was determined in 6 myxedematous females and 5 myxedematous males during hospitalization, and the excretion rates calculated. No attempt was made to select these patients according to serum uric acid levels. In 5 of the 11 myxedematous patients from whom urine specimens were collected, studies were made before and during therapy with thyroactive compounds. Two of these patients were studied while receiving constant repetitious diets. Urate excretion was also determined in 7 hyperthyroid patients, and excretion rates were established in 5.

All of the treated cases of myxedema were given triiodothyronine (T_3) or triiodothyropropionic acid (T_3 Prop). The latter compound is about one-tenth as active as thyroxine in maintaining a normal oxygen consumption in athyrotic subjects (3).

RESULTS

In Figure 1 the serum uric acid values in a group of myxedematous females and males are compared with those in a group of normal subjects. The mean level (and standard deviation) of serum uric acid was 5.2 ± 0.7 mg. per 100 ml. for normal males, and 4.2 ± 0.7 mg. per 100 ml. for normal females. The mean level of serum uric acid for all myxedematous males was 7.2 mg. per 100 ml., which is almost three standard deviations removed from the normal mean. In 11 of the 12 myxedematous males, the serum uric acid level was higher than 6 mg. per 100 ml. The mean value for all myxedematous females was 4.8 mg. per 100 ml., and in only 6 of the 16 females was the level higher than 5.0 mg. per 100 ml. Table 1A shows the data on all the myxedematous female patients. It will be noted that the only females with elevated values were over 60 years of age or had undergone the menopause or oophorectomy several years prior to observation. The levels of blood urea nitrogen in the myxedematous females with elevated levels of serum uric acid did not differ from those in the myxedematous females with normal levels of uric acid. Table 1B shows the data on the myxedematous males.

In Table 2 are listed the data on all patients, both myxedematous and hyperthyroid, in whom 24-hour urinary urates were determined. In some instances the serum uric acid levels were not determined during the periods of urine collection, and the 24-hour excretion rates were therefore not calculated. A definite difference between the hyperthyroid and myxedematous groups can be observed in regard to the average daily urate excretions and the renal urate excretion rates. The normal urate excretion with a low purine diet is about 400 mg. per day, according to Gutman and Yu (4). The average value for daily urate excretion in these myxedematous patients tended to be less than 400 mg.; excretion was higher in the hyperthyroid patients.

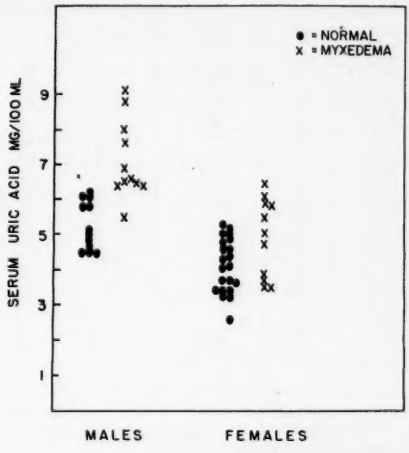

FIG. 1. Comparison of the serum uric acid levels in normal persons and in myxedematous patients.

Table 3 shows the serum uric acid values in several myxedematous patients before and during treatment with either triiodothyronine (T_3) or triiodothyropropionic acid (T_3 Prop.). In each case, enough of the compound was given to bring the basal metabolic rate to a normal level. The average fall in the level of serum uric acid was 2 mg. per 100 ml.

In Figure 2 may be seen the results of the administration of T_3 to a 53-year-old man who had been hypophysectomized eight months previously for metastatic carcinoma of the thyroid. He was myxedematous at the time of the study, and was maintained with 50 mg. of cortisone per day. He was given a moderately low purine diet and his condition equilibrated for several days before he was given a constant repetitive diet. There was a prompt decrease in the serum uric acid level after therapy with triiodothyronine was instituted. This was accompanied by an increase in urate excretion. The average excretion rate before therapy was 1.3 ml. per

minute, but during maintenance with 50 micrograms of triiodothyronine a day it was 3.6 ml. per minute.

Figure 3 also demonstrates the effect of triiodothyronine on the serum uric acid level and the excretion of urates in a myxedematous patient. This 48-year-old woman had metastatic carcinoma of the breast and had been

TABLE 1. SERUM URIC ACID AND BLOOD UREA NITROGEN (BUN) LEVELS IN MYXEDEMATOUS PATIENTS

A. Myxedematous Females

Patient	Age (yrs.)	BUN (mg./100 ml.)	Serum uric acid (mg./100 ml.)	Time (yrs.) after oophorectomy or menopause*	Etiology of myxedema	Other diagnoses
L.N.	50	12.5	3.7	4 (ooph.)	Thyroidectomy	Carcinoma (Ca.), type unknown
J.E.	72	18.2	4.7	10+	Thyroidectomy	Anemia; chronic blood loss
M.G.	57	14.2	4.6	10	Primary	
F.G.	55		3.5	5 (ooph.)	Thyroidectomy	
J.L.	70	15	4.2	10+	Primary	ASHD†
P.M.	61	18.0	4.7	10+	I^{131} therapy	Ca. of thyroid
D.L.	77	18.1	7.1	10+	I^{131} therapy	ASHD; diabetes
E.C.	46	21.2	6.0	10 (ooph.)	Thyroidectomy	Ca. of thyroid
S.R.	58		5.8	10+	Hypophysectomy	Ca. of thyroid
B.H.	59	11.2	5.7	10+	Primary	ASHD
E.M.	50		3.7	0	Thyroidectomy	
M.M.	42		3.8	0	Thyroidectomy	
B.S.	70	12.9	5.8	10 (ooph.)	Thyroidectomy	Ca. of thyroid
M.Z.	43		3.8	4 (ooph.)	Thyroidectomy	
B.S.	38		3.8	0	Thyroidectomy	Ca. of thyroid
L.L.	71	15.4	6.1	10+	I^{131} therapy	ASHD
		Av. 15.7	*4.8*			

* Except when otherwise noted ("ooph."), the 10 and 10+ notations refer to the number of years after the menopause.

† Atherosclerotic heart disease.

B. Myxedematous Males

Patient	Age (yrs.)	BUN (mg./100 ml.)	Serum uric acid (mg./100 ml.)	Etiology of myxedema	Other diagnoses
J.R.	66	10.4	6.6	Thyroidectomy	
J.L.	76	29	8.1	Primary	ASHD, gout, Ca. of tongue
M.A.	77	18	7.6	Thyroidectomy	Emphysema, Ca. of thyroid, ASHD
L.S.	68		8.8	Thyroidectomy	ASHD
J.M.	53	26	9.4	I^{131} therapy	Hypophysectomy, Ca. of thyroid
L.S.	73	15.5	6.4	Primary	ASHD
F.V.	67		6.9	X-ray therapy	
V.L.	57	14.8	6.5	Primary	ASHD, Ca. of colon
E.W.	68	24	8.1	Primary	ASHD, gout
A.W.	46		6.5	X-ray therapy	Ca. of thyroid
C.M.	72	15.5	5.5	I^{131} therapy	Ca. of thyroid
J.B.	74	21.2	6.4	Thyroidectomy	
		Av. 19.4	*7.2*		

TABLE 2. SERUM URIC ACID AND BLOOD UREA NITROGEN LEVELS, AND URINARY EXCRETION OF URATES IN HYPOTHYROID AND HYPERTHYROID PATIENTS

Patient	Age (yrs.)	BUN (mg./100 ml.)	Serum uric acid (mg./100 ml.)	Urinary urates		Diet†	Other diagnoses
				mg./24 hrs.	excretion rate*		
			Myxedema—Males				
J.M.	53	26	9.1	147	1.9	M	Ca. of thyroid; hypophysectomy
E.W.	68	24	8.1	226	2.3	R	ASHD, gout
M.A.	77	18	7.6	242	1.5	R	Emphysema, ASHD
J.B.	74	21.2	6.4	298	3.0	R	
C.M.	72	15.5	5.5	468	6.0	R	
		Av. 20.9	*7.3*	*276*	*2.9*		
			Myxedema—Females				
D.L.	77	18	7.1	322	3.2	R	ASHD, diabetes
P.M.	61	18	4.7	343	5.1	L	ASHD
L.N.	50	12.5	3.7	324	6.5	M	Cancer
J.E.	72	18.2	4.7	223	3.2	M	Anemia
M.G.	57	14.0	4.6	216	3.3	R	
E.C.	46	21.2	6.0	475	6.0	M	Ca. of thyroid
		Av. 17.0	*5.1*	*317*	*4.5*		
			Hyperthyroidism—Both Sexes				
C.P. ♂	53	12.2	4.3	990	16.0	R	
B.M. ♀	44			598		R	
L.L. ♀	61	12.0	5.2	672	9.0	L	Pneumonia
M.L. ♀	64	11.5		590		L	
F.W. ♀	48	10.2	4.3	690	11.1	M	
P.P. ♀	45	10.8	5.2	740	8.4	L	
C.K. ♂	48	18.0	5.3	833	10.6	L	
		Av. 12.4	*4.9*	*730*	*11.0*		

* The urate excretion rate (ml. of serum cleared/min.)

$$= \frac{\text{mg. urate/24 hours}}{\text{mg./ml. serum uric acid}} \Big/ \text{1440 minutes.}$$

† See text for description.

TABLE 3. SERUM URIC ACID LEVELS BEFORE AND DURING THYROID HORMONE THERAPY IN MYXEDEMATOUS SUBJECTS

Patient	Sex	Before therapy (mg./100 ml.)	Daily dose of thyroid hormone	During therapy (mg./100 ml.)
D.L.	F	7.1	T_3 Prop., 2.0 mg.	5.2
L.S.	M	6.4	T_3 Prop., 2.0 mg.	4.8
F.V.	M	6.9	T_3 Prop., 1.5 mg.	4.2
V.L.	M	6.5	T_3 Prop., 1.5 mg.	6.0
B.H.	F	5.7	T_3 Prop., 2.0 mg.	3.4
J.L.	M	8.0	T_3 Prop., 1.5 mg.	6.6
B.S.	F	5.8	T_3 75 μg.	4.3
E.W.	M	8.1	T_3 Prop., 2.0 mg.	5.0
J.M.	M	9.1	T_3 100 μg.	5.6
J.B.	M	6.8	T_3 50 μg.	4.2
C.M.	M	5.5	T_3 75 μg.	4.8
		Av. 6.9		*Av. 4.9*

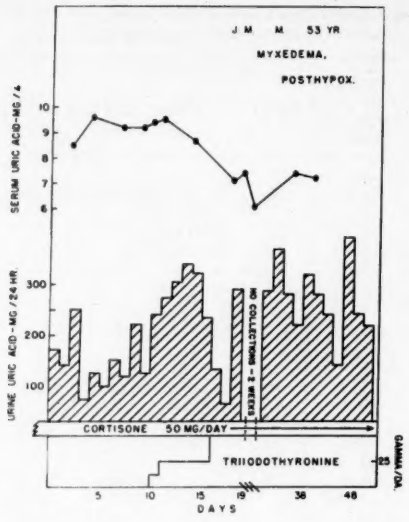

FIG. 2. Effect of triiodothyronine on urate excretion and serum uric acid levels in a hypophysectomized myxedematous male.

surgically castrated six months before the study. Determination of the 24-hour urinary excretion of steroids revealed minimal amounts of 17-ketosteroids (1.2 mg.) and 17-hydroxysteroids (0.9 mg.). Urinary FSH could not be detected in any dilution. Diabetes insipidus was present, and results

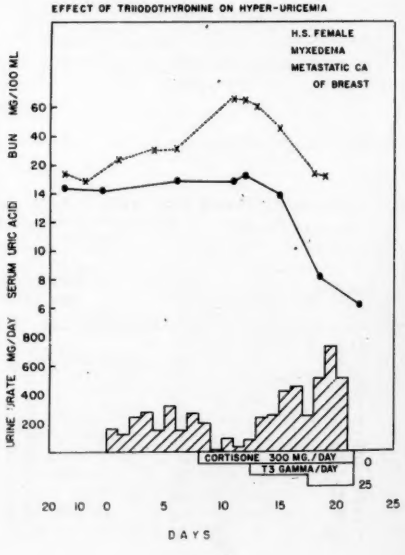

FIG. 3. The uricosuric effect of triiodothyronine, in association with an effect on the level of blood urea nitrogen.

of the Hickey-Hare test were positive. Because of the foregoing findings it was thought that the patient had metastatic involvement of the pituitary in spite of normal roentgenograms and normal visual fields. Cortisone was started in a dosage of 300 mg. per day, and the previously normal urinary volume fell to 200–300 ml. a day. Five days later, triiodothyronine was administered, with the results shown in Figure 3. The excretion rate in this patient increased from 0.8 ml. per minute to 6.8 ml. per minute. The serum uric acid level of 14.1 mg. per 100 ml. is not included in Table 1 or Figure 1 because of the complicating factors.

Figure 4 depicts the results of the administration of triiodothyropro-

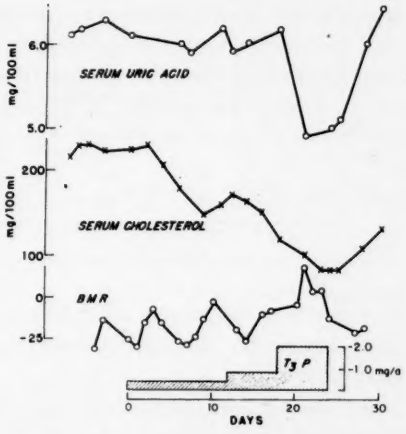

FIG. 4. Effect of increasing dosages of triiodothyropropionic acid on the serum uric acid and cholesterol levels, and on the basal metabolic rate. The patient was receiving a constant repetitive diet.

pionic acid in dosages up to 2 mg. daily in a 68-year-old man with primary myxedema and gout, while he was receiving a repetitious regular hospital diet (R). Effects on the serum cholesterol and uric acid levels were demonstrated, along with an eventual effect on the basal metabolic rate. Rebound was prompt after T_3 Prop. was stopped. Although the cholesterol level decreased during administration of the smaller amounts of T_3 Prop., no effect was demonstrated on the serum uric acid level until doses were given which raised the basal metabolic rate to normal. Interestingly, a classic episode of acute gout and also angina developed when the dosage of the drug was 2 mg. daily.

Figure 5 demonstrates the effect of T_3 Prop. in amounts less than adequate to elevate the basal metabolic rate to normal. With such dosages there was an early increase in daily urate excretion, but the increase was not maintained. In none of the 3 patients was the concentration of serum uric acid altered.

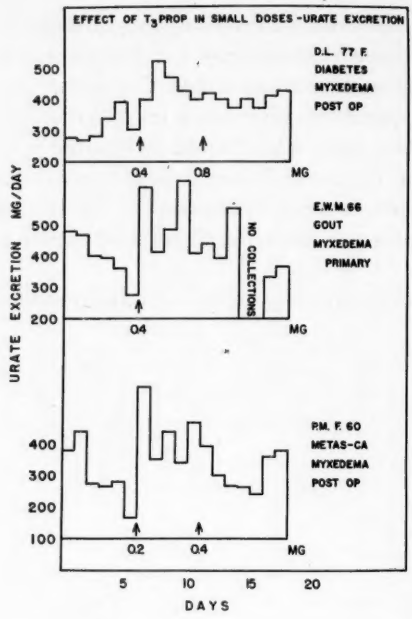

FIG. 5. Small doses of triiodothyropropionic acid caused an early but unsustained increase in urinary urates.

DISCUSSION

Little evidence has been previously advanced that the metabolism or excretion of uric acid is affected by thyroid function in the human subject.

Rawson and co-workers (5) noted no change in urate excretion after single intravenous injections of large doses of thyroxine or of triiodothyronine. We noted no changes in urate excretion after a single intravenous dose of 10 mg. of T_3 Prop. There was, however, a moderate increase in urinary urates after the intravenous administration of 0.5 mg. of T_3 in 1 patient. All changes described in this study were produced by daily oral medication.

An association between thyroid function and uric acid metabolism was suggested by the data of Kuzell *et al.* (6). In 520 cases of gout, these authors found a 30 per cent incidence of hypothyroidism in females and a 20 per cent incidence in males. The well known fact that males and postmenopausal women are much more prone to suffer from gout is interesting in the light of the facts brought out in our studies. In the present investigation the incidence of hyperuricemia was much higher in the males and in the late postmenopausal female.

Hyperuricemia in hypoparathyroidism has been observed by Dubin *et al.* (7). Nine of the 11 cases of hypoparathyroidism described in their report were post-thyroidectomy. Although many of our patients had had thyroidectomies, none was hypoparathyroid by clinical or laboratory criteria. Hyperuricemia has been observed in arteriosclerotic heart disease by Gertler and White (8). Many of their patients also had evidence of moderately decreased thyroid function.

Capra (9) found that administration of large doses of thyroxine decreased urate excretion in euthyroid patients with liver disease. This might seem to contradict our findings, but since the patients studied were ex-

creting large amounts of uric acid (more than 1 Gm. per day), and since they did have liver disease, no comparisons can be made. The method for determining urinary urates may also have affected the results.

The foregoing indirect evidence suggests a possible relationship between thyroid function and uric acid metabolism or excretion in the human. In the rat (10, 11) it has been shown that urate excretion increases 40–50 per cent when large amounts of desiccated thyroid are given. In one of the studies, the serum uric acid level also rose 40–50 per cent, and in both studies there was a parallel increase of nitrogen in the urine.

The present data indicate that the effect of thyroid hormone on uric acid in myxedematous patients is mediated primarily through an effect on the kidney. The changes in 24-hour excretion rates and total urate excretion before and during therapy, and the differences in urate excretion rate and total urate excretion between the hyperthyroid and the hypothyroid patients strengthen this thesis. The moderately increased level of blood urea nitrogen in most of the myxedematous patients also is in favor of a primary renal effect.

Because many of the patients reported here had cancer of various types, it is possible that in some instances the hyperuricemia may have been related to the destruction of tissue and increased nucleic acid catabolism. However, especially in the male patients, hyperuricemia was apparent regardless of the presence or absence of cancer. In addition, only a few of these patients had active progressive disease. Even if the hyperuricemia were in some cases related to the presence of cancer, a hypo-uricemic effect was demonstrated when thyroactive substances were given to myxedematous patients.

Although the hypo-uricemic response that occurs when thyroactive substances are given to myxedematous patients is in all likelihood caused by an increase in renal blood flow, the possibility exists that larger doses of thyroid may increase purine turnover rates. The somewhat elevated excretion of uric acid in the hyperthyroid patients reported here, and in animal studies, suggests the latter possibility, which is being investigated by means of C^{14}-labeled glycine.

REFERENCES

1. Forsham, P. H.; Thorn, G. W.; Prunty, F. T. G., and Hills, A. G.: Clinical studies with pituitary adrenocorticotropin, *J. Clin. Endocrinol.* **8:** 15, 1948 (footnote 8, p. 20).
2. Kern, A., and Stransky, E.: Beitrag zur kolorimetrischen Bestimmung der Harnsäure, *Biochem. Ztschr.* **290:** 419, 1937.
3. Benua, R. S.; Leeper, R. D.; Kumaoka, S., and Rawson, R. W.: Metabolic effects of thyroxine analogues in human myxedema, *Ann. New York Acad. Sc.* **86:** 563, 1960.

4. GUTMAN, A. B., and YU, T. F.: Renal function in gout, *Am. J. Med.* **23**: 600, 1957.
5. RAWSON, R. W.; RALL, J. E.; PEARSON, O. H.; ROBBINS, J.; POPELL, H. F., and WEST, C. D.: *l*-Triiodothyronine versus *l*-thyroxine; a comparison of their metabolic effects in human myxedema, *Am. J. M. Sc.* **226**: 405, 1953.
6. KUZELL, W.; SCHAFFARZICH, R. W.; NAUGLER, E. W.; KOETS, P.; MANKLE, E. A.; BROWN, B., and CHAMPLIN, B.: Some observations on 520 gouty patients, *J. Chronic Dis.* **2**: 645, 1955.
7. DUBIN, A.; KUSHNER, D. S.; BRONSKY, D., and PASCALE, L. R.: Hyperuricemia in hypoparathyroidism, *Metabolism* **5**: 703, 1956.
8. GERTLER, M. M., and WHITE, P. D.: Coronary Artery Disease in Young Adults: A Multidisciplinary Study. Cambridge, Harvard University Press, 1954.
9. CAPRA, P.: Effect of thyroxin on blood and urinary uric acid, *Arch. sc. med.* **93**: 352, 1952.
10. BROWN, E. F., and MORGAN, A. F.: Nitrogen metabolism of the normal and the vitamin A-deficient rat as affected by thyroid administration, *J. Nutrition* **35**: 439, 1948.
11. SURE, B.; FORD, Z. W., JR.; THEIS, R. M., and GOLDFISCHER, M.: Nitrogen metabolism in hyperthyroidism, *Endocrinology* **28**: 806, 1941.

UPTAKE OF I^{131}-*l*-TRIIODOTHYRONINE IN VARIOUS ERYTHROCYTE ABNORMALITIES

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ABSTRACT

This study was undertaken to determine whether the erythrocyte uptake of I^{131}-*l*-triiodothyronine is affected by various erythrocyte abnormalities and whether consistent variations occur in association with several abnormal hematologic conditions. Plasma protein-bound iodine levels were determined in most cases in an attempt to rule out unsuspected thyroid disease. Normal I^{131}-*l*-triiodothyronine uptake values ranged from 10.58 to 18.34 per cent. In 3 cases of sickle-cell trait, normal values were observed, whereas in 2 pregnant patients with the trait, uptake values were low. Normal uptakes were also recorded in 1 case of sickle-cell anemia, 1 of sickle-cell thalassemia and 3 of thalassemia trait. Varying results were obtained in 9 cases of hemolytic anemia; in 2 cases associated with acute leukemia the uptakes were elevated despite very low hematocrits; in 3 cases associated with Hodgkin's disease, normal uptakes were noted in 2 and an increased uptake in 1; values were normal in a case of congenital spherocytic hemolytic anemia and elevated in a case of congenital hemolytic anemia with unusual inclusion bodies. Uptakes were elevated in 6 of 8 cases of polycythemia vera, and the value was high-normal in the seventh. Although variations in hematocrit have been shown to affect erythrocyte uptake, this factor did not account for the elevations in this group. No correlation between erythrocyte uptake of I^{131}-*l*-triiodothyronine and hematocrit could be made in the cases of polycythemia vera or in the cases of hemolytic anemia with elevated uptake values.

THE uptake of I^{131}-*l*-triiodothyronine by erythrocytes *in vitro* as a means of assessing thyroid function has been described by several authors. The test is of especial value, since administration of a radioactive substance to the patient is avoided. From the standpoint of accuracy, it compares favorably with other diagnostic procedures (1, 2) and also permits assessment of thyroid function in situations where standard tests cannot be used (2, 3). Although there is good correlation between erythrocyte uptake of I^{131}-*l*-triiodothyronine and thyroid activity, several factors

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have been shown to affect uptake values. Decreased values have been noted in pregnancy and following administration of estrogens and propylthiouracil. Increased values have been noted following administration of anticoagulants and in the presence of severe liver disease, metastatic malignancy, severe pulmonary insufficiency, and paroxysmal atrial arrhythmia (4, 5).

The present study was undertaken to extend our knowledge of factors which might alter uptake values in euthyroid subjects. Specifically, our purpose was to determine whether or not uptake is affected by various erythrocyte abnormalities and whether consistent variations occur in any of the several abnormal conditions studied. No previous observations on altered erythrocyte uptake in hematologic disorders have been reported.

MATERIALS AND METHODS

The method utilized was similar to that described by Hamolsky, Stein and Freedberg (4). A 0.1-ml. sample of I^{131}-*l*-triiodothyronine (1.1×10^{-2} micrograms (μg.) per 0.1-ml.) was added to 3 ml. of heparinized whole blood in a 10-ml. stoppered Erlenmeyer flask and incubated for two hours at 37° C. in a water bath. Two 1-ml. aliquots were then counted in a well-type scintillation counter. A 10-ml. volume of physiologic salt solution was added to each of these whole blood samples. They were then centrifuged at 3,000 rpm for ten minutes, the supernatant removed, the cells washed five times in physiologic salt solution at room temperature, and the radioactivity of the cells measured. The percentage uptake of radioactivity in the samples was then determined and corrected to 100 per cent hematocrit.

Normal values as determined in this clinic are: males, $\bar{\ }\pm 2$ standard deviations $= 14.88 \pm 3.46$; females, $\bar{Y} \pm 2$ s.d. $= 14.14 \pm 3.56$.

All patients were clinically euthyroid. The plasma protein-bound iodine (PBI) level was determined in most cases as a means of ruling out unsuspected thyroid disease. In all patients tested, the PBI level was within the normal range of 3.5–8.0 μg. per 100 ml., except in the women who were also pregnant; in these women, slight elevations were noted (6).

Patients were obtained from the Hematology Service of the Walter Reed General Hospital, and in some instances from the Hematology Clinic, D. C. General Hospital, Washington, D. C.

"Crisscross" experiments (3) were performed in cases of polycythemia vera and acute leukemia with hemolytic anemia. This consisted of interchanging the patient's red blood cells with normal plasma and the patient's plasma with normal red blood cells. Mixing of plasma and cells was carried out so as to maintain the original hematocrits.

RESULTS

A summary of the data is shown in Table 1.

Hemoglobinopathies and thalassemia

Ten cases were studied in this group. Normal values were obtained in 3 cases of sickle-cell trait, 1 of sickle-cell anemia, 1 of sickle-cell thalassemia and 3 of thalassemia trait. In 2 cases of sickle-cell trait associated with pregnancy, the values were low. This is in keeping with previous data on

TABLE 1. UPTAKE OF I^{131}-*l*-TRIIODOTHYRONINE BY RED BLOOD CELLS IN VARIOUS ERYTHROCYTE ABNORMALITIES

Diagnosis	RBC uptake of I^{131}-T_3 (%)	Plasma PBI (μg./100 ml.)	Hematocrit (%)	Remarks
Hemoglobinopathies and thalassemia				
1. Sickle-cell trait (A-S)	17.8	6.4	45	
2. Sickle-cell trait (A-S)	12.5	6.0	39	
3. Sickle-cell trait (A-S)	18.1	—	23	
4. Sickle-cell trait (A-S)	9.4	8.1	26	7 months pregnant.
5. Sickle-cell trait (A-S)	6.9	9.0	32	6 months pregnant.
6. Sickle-cell anemia (S-S)	16.2	7.1	20.5	
7. Sickle-cell-thalassemia (S-A-F)	11.7	5.8	38	
8. Thalassemia trait	13.3	7.1	46	
9. Thalassemia trait	14.1	—	44	
10. Thalassemia trait	14.1	6.0	41	
Hemolytic anemias				
1. Idiopathic hemolytic anemia	15.8	6.4	38	
2. Congenital hemolytic anemia with inclusion bodies (7)	25.8	—	35	PBI not determined; no clinical evidence of hyperthyroidism.
3. Hodgkin's disease with hemolytic anemia	16.8	5.0	22.5	
4. Hodgkin's disease with hemolytic anemia	13.0	—	22	
5. Hodgkin's disease with hemolytic anemia	19.9	—	33	No clinical evidence of hyperthyroidism.
6. Paroxysmal nocturnal hemoglobinuria	10.3	—	19	
7. Hereditary spherocytic hemolytic anemia	16.5	—	34	
8. Acute leukemia with hemolytic anemia	20.6	—	15.5	No clinical evidence of hyperthyroidism.
9a. Acute leukemia with hemolytic anemia	29.6	3.8	13.5	Original hematocrit.
b. Same as above	32.9		25	Doubling hematocrit caused only slight change in uptake.
Polycythemia vera				
1.	16.5	7.3	50	
2.	25.5	5.8	50	
3.	22.3	5.5	49	Severe pulmonary emphysema.
4.	21.6	3.7	48.5	
5.	19.7	6.2	59	
6.	20.8	4.1	43	
7.	22.0	5.0	67	
8.	19.0	5.5	48	

pregnant women (3). The results suggest that uptake values are not affected by the sickle type of hemoglobinopathy or by the thalassemia trait.

Hemolytic anemia

Several types of hemolytic anemia were studied (9 cases). Normal values were obtained in 1 case of congenital spherocytosis and 1 of idiopathic acquired hemolytic anemia. An elevated value was found in 1 case of congenital hemolytic anemia with abnormal pigment metabolism and

red-cell inclusion bodies (7). A low-normal level was recorded in 1 case of paroxysmal nocturnal hemoglobinuria. Three cases of symptomatic hemolytic anemia secondary to Hodgkin's disease were included in the group; in 2 the uptake was normal, and in 1 it was elevated. In the 2 cases of acute leukemia in adults with associated hemolytic anemia there was a significant elevation in uptake.

Polycythemia vera

Eight subjects were included in this group. The diagnosis of polycythemia vera was certain in 7; the remaining case probably represented the primary variety. This case (No. 3) was originally diagnosed as polycythemia vera, but the presence of significant pulmonary emphysema made the diagnosis uncertain. In 7 of these 8 patients, elevated uptake values were observed.

DISCUSSION

These results indicate that abnormalities of the *in vitro* erythrocyte uptake of I^{131}-*l*-triiodothyronine may occur in the absence of thyroid disease or of other conditions in which abnormal uptakes have previously been demonstrated.

No unusual values were noted in any of the cases of hemoglobinopathy or thalassemia trait, except for low uptakes in 2 cases associated with pregnancy. This suggests that the test can be performed in these conditions without fear of abnormal results due to the hematologic disorder.

The findings in the cases of hemolytic anemia were less consistent. In 6 of the 9 patients, the uptakes were normal. The elevated uptakes in the 2 cases of acute leukemia were of interest, especially in view of the low hematocrit values in both cases. As previously shown by Crispell and associates (8), the presence of plasma markedly decreases the uptake by red cells in a saline system. Hamolsky and co-workers (2) noted variable results in specimens of whole blood with hematocrit values outside the range of 30–55 per cent. We have been able to show consistent variations in erythrocyte uptake by *in vitro* manipulation of the hematocrit in normal subjects, as shown in Figure 1. This change in uptake can probably be explained by the variations in the amount of plasma thyroxine-binding protein that occur with changes in hematocrit. Therefore, if the changes in uptake noted in these leukemia cases were due to changes in hematocrit, decreased uptakes (increased amounts of plasma thyroxine-binding protein) would have been expected. In an attempt to localize the site of the increased uptake, a crisscross experiment was performed, as shown in Figure 2. The results suggest that a plasma factor accounts for the elevated uptake, possibly due to a decrease in thyroxine-binding protein as a

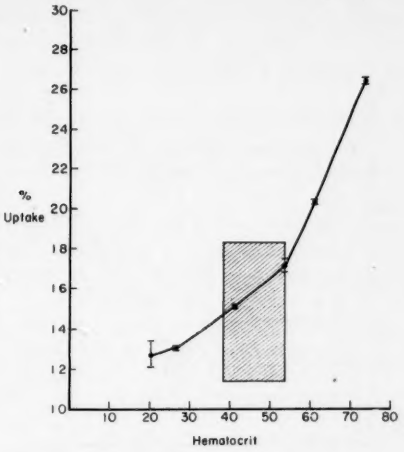

FIG. 1. Variation in red blood cell uptake of I^{131}-*l*-triiodothyronine in a normal subject, caused by *in vitro* alterations in the hematocrit. The normal range is shown by the shaded area.

result of the disease. The reason for an increased uptake in only 1 of the 3 cases of hemolytic anemia secondary to Hodgkin's disease is not clear.

The most consistent results were obtained in cases of polycythemia vera. In this group, elevated uptake values were found in 7 of 8 cases. Elevated values might have been expected on the basis of increased hematocrits and resultant decreased amounts of thyroxine-binding protein. That the elevations cannot be explained by this factor alone is evident from Figure 3. It can be seen that most of the increased uptakes occurred in patients with a normal or high-normal hematocrit at the time of the study.

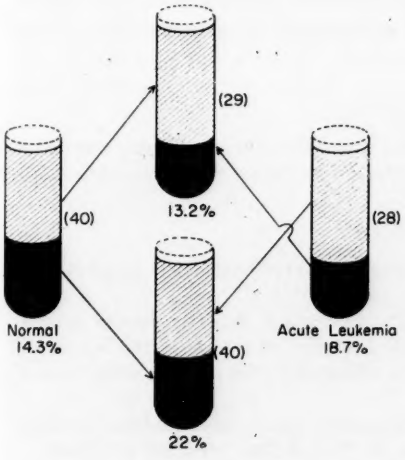

FIG. 2. Crisscross experiment utilizing normal and leukemic blood. Values for I^{131}-*l*-triiodothyronine uptake are expressed as percentages, and hematocrit values are shown in parenthesis.

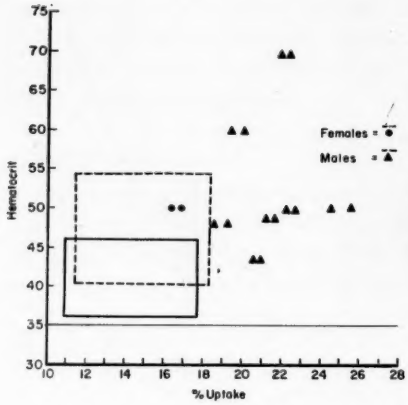

FIG. 3. Relationship of hematocrit to I^{131}-*l*-triiodothyronine uptake in erythrocytes of patients with polycythemia vera. Normal ranges are shown as blocked areas.

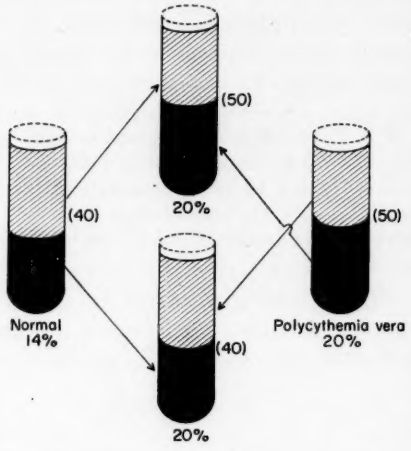

FIG. 4. Crisscross experiment utilizing normal and polycythemic blood. Values for I^{131}-*l*-triiodothyronine uptake are expressed as percentages, and hematocrit values are shown in parenthesis.

A "crisscross" study performed in a case of polycythemia yielded surprising results, as shown in Figure 4. Increased uptake was induced by adding polycythemic plasma to normal red blood cells, and similar levels were noted with normal plasma added to polycythemic red blood cells. This observation was made on two separate occasions. Whether this increase in the presence of cells from polycythemic plasma was due simply to the increased number of cells or to some intrinsic abnormality of the cells is not clear.

Acknowledgment

The authors are indebted to Dr. Paul McCurdy, Hematology Clinic, District of Columbia General Hospital, for providing several of the blood specimens used in the study.

REFERENCES

1. BAUER, R. E.: The present status of the diagnosis of hyperthyroidism, *Ann. Int. Med.* **44:** 207, 1956.
2. HAMOLSKY, M. W.; GOLODETZ, A., and FREEDBERG, A. S.: The plasma protein-thyroid hormone complex in man. III. Further studies on the use of the *in vitro* red blood cell uptake of I^{131}-*l*-triiodothyronine as a diagnostic test of thyroid function, *J. Clin. Endocrinol. & Metab.* **19:** 103, 1959.
3. URELES, A. L., and MURRAY, M.: The erythrocyte uptake of I^{131}-labeled *l*-triiodothyronine as a measure of thyroid function, *J. Clin. & Lab. Med.* **54:** 178, 1959.

4. HAMOLSKY, M. W.; STEIN, M., and FREEDBERG, A. S.: The thyroid hormone-plasma protein complex in man. II. A new *in vitro* method for study of "uptake" of labelled hormonal components by human erythrocytes, *J. Clin. Endocrinol. & Metab.* **17**: 33, 1957.
5. KURLAND, G. S.; GOLODETZ, A.; HAMOLSKY, M. W., and FREEDBERG, A. S.: Thyroid function in supraventricular tachycardias: turnover of intravenously infused I^{131}-labeled thyroxine and the red blood cell uptake of I^{131}-labeled-*l*-triiodothyronine, *J. Clin. Endocrinol. & Metab.* **19**: 92, 1959.
6. WERNER, S.: The Thyroid, ed. 1. New York, Paul B. Hoeber, Inc., 1955, p. 142.
7. LANGE, R. D., and AKEROYD, J. H.: Congenital hemolytic anemia with abnormal pigment metabolism and red cell inclusion bodies: a new clinical syndrome, *Blood* **13**: 950, 1958.
8. CRISPELL, K. R.; KAHANA, S., and HYER, H.: The effect of plasma on the *in vitro* uptake or binding by human red cells of radioactive I^{131}-labeled-L-thyroxine and L-triiodothyronine, *J. Clin. Invest.* **35**: 121, 1956.

THE *IN VITRO* RESIN SPONGE UPTAKE OF TRIIODOTHYRONINE-I^{131} FROM SERUM IN THYROID DISEASE AND IN PREGNANCY*

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ABSTRACT

A procedure has been devised which measures the uptake of I^{131}-labeled triiodothyronine (T_3-I^{131}) from serum *in vitro* by means of a resin sponge. The sponge, consisting of a mixture of polyurethane foam and a finely divided anion exchange resin (Amberlite IRA-400), was equilibrated with serum containing T_3-I^{131}. Significant differences in the sponge uptake of T_3-I^{131} from serum were demonstrated in hyperthyroidism, myxedema, and uncomplicated pregnancy when compared with the sponge uptake from the serum of normal subjects; values were elevated in hyperthyroidism and reduced in pregnancy and myxedema. Augmented sponge binding of T_3-I^{131} was also observed following the administration of thyrotropin to subjects other than those with primary myxedema. The results indicate that the resin sponge procedure may be a useful means for distinguishing between certain thyroidal abnormalities and for recognizing pregnancy and pregnancy complicated by hyperthyroidism.

PREVIOUS studies have demonstrated distinct differences in the resin adsorption of I^{131}-labeled thyroxine from the serum of pregnant women (1), as well as from patients with hyperthyroidism and myxedema (2). However, precise delineation of these conditions on the basis of clearly defined values proved impractical because of the sporadic fluctuations in the differences. Therefore, the usefulness of the procedure as a diagnostic aid in thyroid disease was limited. Subsequent studies indicated that the fluctuations were probably related to some of the intrinsic properties of the granular type resins. Hence, other physical forms of anion exchange resins were examined for their thyroxine-binding and triiodothyronine-binding characteristics. Eventually, a resinous material, indistinguishable from plastic sponge, was found to provide consistently uniform values. This report describes the procedure and the results obtained in the meas-

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urement of triiodothyronine binding by sera from several different groups of subjects.

MATERIALS AND METHODS

The steps of the procedure are outlined in Figure 1. The resin sponge was prepared commercially[1] by mixing polyurethane foam with a finely ground anion exchange resin, Amberlite IRA-400 (200–400 mesh) in the chloride cycle. The resin constituted 28.6 to 29.5 per cent of the weight of the sponge. Individual plugs of sponge measuring 1.2 cm. ×1.2 cm. (50 to 60 mg.) were placed in test tubes (16 mm. ×100 mm.) containing 2 ml. of tris-maleate buffer,[2] pH 5.2 (0.2*M*). The tubes containing the buffer and the sponges were set aside for one-hour to four-hour periods before they were used.

Sera were obtained from healthy volunteers, convalescing patients, patients with unequivocal hyperthyroidism or myxedema, and women with uncomplicated pregnancies. The sera were stored frozen at 4° C., and frequently the same serum underwent repeated freezing and thawing before use.

A 2-ml. aliquot of serum was equilibrated approximately thirty minutes with 0.01 ml. of 1 per cent albumin solution containing approximately 0.05 μc. of I^{131}-labeled triiodothyronine[3] (T_3-I^{131}) and 0.002 to 0.0008 μg. of stable triiodothyronine. Duplicate 1-ml. aliquots of the serum were added to the tubes which contained the buffer and the sponges. The stoppered tubes, inclined at a 30° angle to the horizontal, were shaken for one hour at 100 to 120 cycles per minute at room temperature (usually 23° C.). Following this, the radioactivity within the tubes was measured in a well-type scintillation detector. The sponges, while in the tubes, were washed three to four times by filling the tube completely with distilled water and then aspirating the supernatant solution. After the final washing, sufficient distilled water was added to the tube to equal the starting volume and the radioactivity was measured again. The fraction of T_3-I^{131} bound by the sponge was calculated by dividing the residual radioactivity by the initial radioactivity, and multiplying the product by 100. The average of the duplicate values was used.

RESULTS

Duplicate determinations

The mean difference between the duplicate values was 1.7 ± 1.3 per cent, and the duplicate variability for 95 per cent of the determinations was 4.2 per cent. Duplicate determinations of borderline values which did not agree within 3 per cent were repeated. Slight variations in sponge weights (50 to 60 mg.) appeared to have no effect upon the duplicate measurements.

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[1] The authors are grateful to Dr. Byron T. Eberle of Abbott Laboratories for providing the resin sponge (Triosorb).

[2] Tris(hydroxymethyl)aminomethane (tris-maleate) buffer, used originally because of its stabilizing influence with the granular resins. It is possible that physiologic salt solution could be substituted for the tris-buffer without changing the results.

[3] Triomet (Abbott Laboratories, Oak Ridge, Tennessee). Specific activity of the shipments ranged from 24.0 mc. per mg. to 67.0 mc. per mg. From 1 to 3 ml. of 1 per cent human serum albumin was added to the shipment vial, which was stored at 4° C.

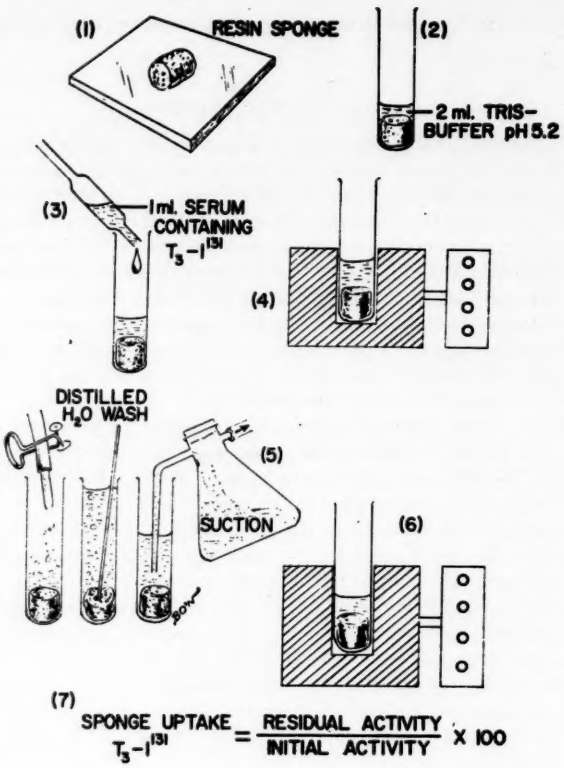

FIG. 1. Procedure for resin sponge uptake of T_3-I^{131}. Steps are outlined in numerical sequence.

Bond between sponge and T_3-I^{131}

In order to be sure that the bond between the sponge and the T_3-I^{131} was constant, it was necessary to ascertain whether or not the uptake values would change with repeated washing. Following equilibration with T_3-I^{131}, the sponges were washed repeatedly with distilled water, and the uptake of T_3-I^{131} was determined after each single washing. It was apparent from the results (Fig. 2) that the T_3-I^{131} not bound by the sponge was removed with the first three washings and that further washing had no effect upon the radioactivity remaining on the sponge.

Influence of the medium

I^{131}-labeled T_3 diluted in phosphate buffer or 4 per cent serum albumin was taken up almost entirely by the sponge, whereas only a small fraction of T_3-I^{131} in normal serum was bound by the sponge (Fig. 3).

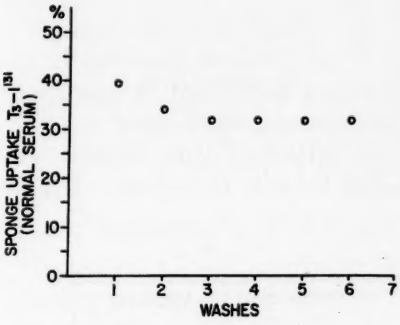

FIG. 2. Effect of repeated washing upon the resin sponge uptake of T_3-I^{131} from serum. After the first three washings the value remained constant.

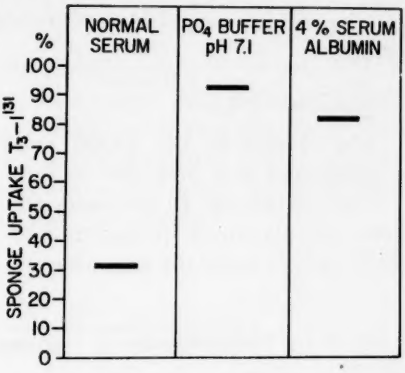

FIG. 3. Influence of the medium upon the resin sponge uptake value. Sponge binding of T_3-I^{131} was reduced in serum compared with other media.

Slight changes in room temperature had no discernible effect upon the sponge uptake of the labeled T_3, and moderate elevations in temperature produced only slight increments in the T_3-I^{131} uptake (Fig. 4)

It was apparent that the time allowed for equilibration between sponge and serum determined to a great extent the magnitude of the binding values (Fig. 5). Within the first two hours the fraction of T_3-I^{131} adsorbed

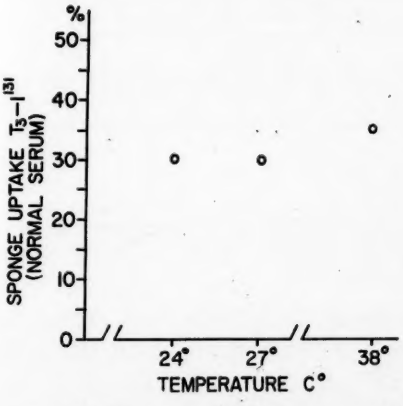

FIG. 4. Effect of temperature upon the resin sponge uptake of T_3-I^{131} from serum. Small increments in uptake occurred in association with elevated temperatures.

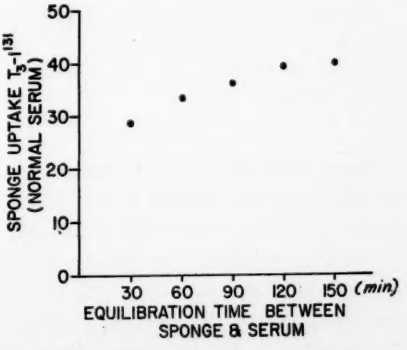

FIG. 5. Change in resin sponge uptake of T_3-I^{131} correlated with duration of equilibration between sponge and serum. Within the first two hours the magnitude of the uptake varied almost directly with the duration of equilibration.

by the sponge varied almost directly with the time of exposure to the serum.

Group determinations

The results of the group determinations in normal, hyperthyroid, hypothyroid, and pregnant subjects are illustrated in Figure 6.

Normal subjects. In 109 determinations carried out on 65 normal volunteers, the one-hour sponge uptakes ranged from 25 to 35 with a mean of 30.1 ± 2.8 (1 standard deviation).

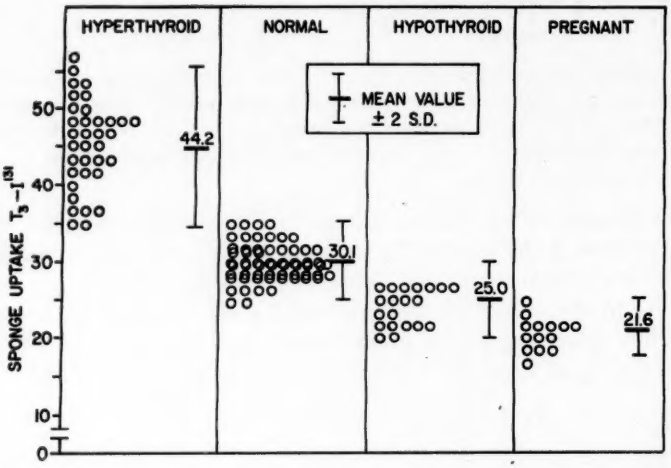

FIG. 6. Resin sponge uptake of T_3-I^{131} from sera of different groups of subjects. Each circle represents the uptake value for a single subject. The solid bar indicates the mean value for the group. The lines extending above and below the bar encompass two standard deviations.

Hyperthyroidism. In 35 determinations on 34 hyperthyroid patients, the sponge uptakes ranged from 35 to 57 with a mean of 44.2 ± 5.9 ($P < 0.001$ compared with the normal value).

Hypothyroidism. In 20 determinations on 19 patients with myxedema the uptakes ranged from 20 to 27 with a mean of 25.0 ± 2.5 ($P < 0.001$ compared with the normal value).

Pregnancy. In 16 determinations on 14 women with uncomplicated pregnancies the uptakes ranged from 18 to 25 with a mean of 21.6 ± 2.6 ($P < 0.001$ compared with the normal value).

Acute and chronic illnesses. In 14 determinations on 13 patients with a variety of acute and chronic illnesses the uptakes ranged from 26 to 39.

The mean of 33.7 ± 3.7 was noticeably higher than that in the normal controls.

The number of determinations could not be increased because of the limited supply of sponges. Fortunately, the sponges were capable of being used over two or three times without any appreciable reduction in their ability to bind. Since it was not possible to elute entirely the T_3-I^{131} from the sponges, it was necessary to measure the radioactivity remaining from the previous determination before re-using the sponge.

Effect of administration of thyrotropin (TSH)

The effect of TSH was studied in 6 chronically ill patients who had no apparent evidence of thyroid disease and in 1 patient with treated primary

TABLE 1. CHANGES IN THE SPONGE UPTAKE OF T_3-I^{131} AND IN THE SERUM PROTEIN-BOUND IODINE LEVEL FOLLOWING ADMINISTRATION OF THYROTROPIN (TSH)

Patients	Sponge uptake of T_3-I^{131} (%)		Serum protein-bound iodine (μg./100 ml.)*	
	Before TSH	After TSH	Before TSH	After TSH
Convalescent				
G.W.	31.6	39.7	5.2	9.7
M.M.	26.1	36.0	4.2	9.2
E.D.	37.1	52.7	5.4	11.7
C.C.	30.3	41.8	3.7	9.5
G.R.	30.0	42.7	6.2	9.7
G.C.	39.2	52.7		
Primary myxedema				
H.P.	22.4	22.7	4.4	4.5

* Determinations performed by Graham Laboratories, Inc., Brookline, Massachusetts.

myxedema. Serum was obtained from each patient before and after the daily administration of TSH[4] (10 U.S.P. units) for three consecutive days. Changes in the sponge uptake and in the level of serum protein-bound iodine are summarized in Table 1. In each instance, TSH produced significant increments in the sponge binding of T_3-I^{131}, with the single exception of the patient with myxedema.

Effect of thyroxine in vitro

Enrichment of serum with increments of stable thyroxine (T_4) before the addition of the I^{131}-labeled triiodothyronine produced an almost proportional increase in the sponge uptake values (Fig. 7). However, some sera exhibited responses of lesser magnitude in the uptake of T_3-I^{131} under identical conditions of thyroxine enrichment.

It should be noted that I^{131}-labeled thyroxine could be used in place of

[4] Thytropar (Armour).

I^{131}-labeled triiodothyronine, with comparable results in all instances. However, smaller fractions of the T_4-I^{131} were taken up by the sponge and the differences between the group values were not as distinct as they were with I^{131}-labeled T_3.

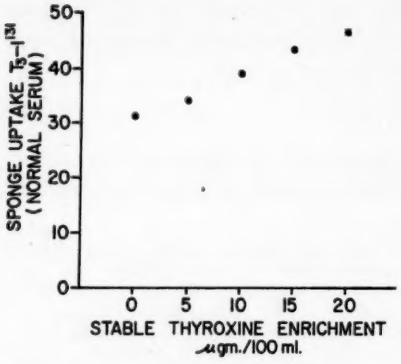

FIG. 7. Effect of increasing concentrations of stable thyroxine upon the resin sponge uptake of T_3-I^{131}. The magnitude of the sponge uptake varied to some extent with the concentration of stable thyroxine

Effect of administration of antithyroid drugs and other materials

The administration of propylthiouracil to hyperthyroid subjects induced prompt reductions in the sponge uptake of T_3-I^{131}. In most instances the diminution in uptake occurred before the patients had shown any sign of clinical improvement.

The effects of inorganic iodide and of iodinated contrast media were studied in only a few patients. The results suggested that these substances may have little effect upon the sponge uptake of T_3-I^{131}.

DISCUSSION

The technique of interaction analysis by the differential resin adsorption of labeled hormones was developed in order to measure thyroxine binding in pregnancy and thyroid disease (3) as well as to quantitate the binding of insulin by serum (4). The procedure may be visualized as one in which there is competition for the hormone between the resin, on the one hand, and the binding sites of the serum proteins, on the other. If one assumes that the resin has a fixed affinity for the molecules in question then the intensity or the extent of the protein binding may be measured. This appears to be the case with reference to resin sponge. In hyperthyroidism apparently there is either a reduction in the quantity of binding protein or a qualitative change in the capacity of the protein to bind triiodothyronine (or thyroxine). This results in a larger fraction of the labeled triiodothyronine being taken up by the sponge. In myxedema, and especially in pregnancy, the converse may be true. The difference in uptake

values between the two latter conditions suggests that the magnitude of protein binding is considerably greater in pregnancy than in hypothyroidism.

The precise nature of the thyroxine-binding protein is not clear. There

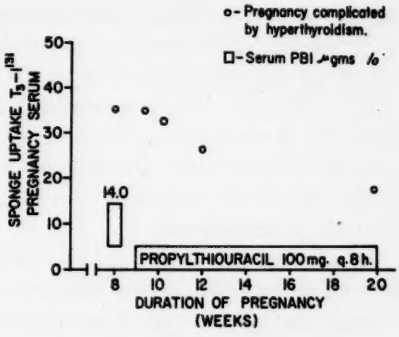

FIG. 8. The resin sponge uptake of T_3-I^{131} in pregnancy complicated by hyperthyroidism. The uptake value at the eighth week of pregnancy was in the high-normal range. During propylthiouracil therapy, the uptake returned to the value ordinarily observed in pregnancy.

is evidence for at least two potent thyroxine-binding fractions in serum (5, 6). One of them, the pre-albumin-binding protein, has been shown to be low in hyperthyroidism as well as in some conditions unrelated to thyroid disease (7). Furthermore, in the same study, a close correlation was established between the decreased binding by the pre-albumin fraction and and the augmented uptake of labeled triiodothyronine by the red blood cell technique (8). This observation is consistent with the results of previous studies with granular type resins, which demonstrated appreciably higher uptakes of labeled thyroxine in some patients suffering from a variety of acute and chronic illnesses (9). Preliminary observations with resin sponge indicate similar results.

It is of interest that the magnitude of the sponge uptake does not appear to be completely dependent upon the concentration of stable thyroxine. Thus, it seems reasonable to believe that the thyroxine-binding protein is the more important consideration with regard to the uptake value. This is evident in the extremely low T_3-I^{131} uptake values in pregnancy[5], a condition in which an elevated level of serum protein-bound iodine is the usual finding. Although hyperthyroidism coincident with pregnancy enhances the sponge uptake of T_3-I^{131}, the augmented value need not exceed the range of high normal (Fig. 8). Since hyperthyroidism occurring in pregnancy is frequently difficult to recognize by the usual clinical and biochemical changes, the unique *in vitro* uptake value obtained with resin sponge should be helpful in arriving at a correct diagnosis.

[5] Sponge uptake values in the range for nonpregnant normal subjects have recently been observed in pregnancy terminating in spontaneous abortion.

The ability of thyrotropin to augment the sponge uptake in subjects with a functioning thyroid gland is not easily explained. It does not seem likely that the increase in serum thyroxine concentration is the sole cause of the elevated uptake, since there is an inconsistent correlation between the serum protein-bound iodine level *in vivo* and the magnitude of the sponge uptake *in vitro*. That a potentially functioning thyroid gland must be present in order for the changes to occur is evident in the lack of change following the administration of TSH in the patient with primary myxedema (Table 1). It is possible that TSH releases some intermediate factor from the thyroid gland which acts specifically upon the thyroxine-binding protein. Conceivably, this pattern of response to TSH could prove to be a useful means for assessing the integrity of the pituitary-thyroid axis in secondary myxedema.

Of all the data obtained, the values in patients with hypothyroidism proved to be troublesome because of the overlap with normal values. Although the number of observations was not great, overlap with normal values was also observed in a larger group of hypothyroid patients when the granular resin uptake of labeled thyroxine was used (9). It would appear that these results are virtually the same as those obtained by means of the erythrocyte uptake of T_3-I^{131} (10, 11). On the other hand, there was little overlap between hyperthyroid and pregnancy values compared to normal values for the sponge uptake of T_3-I^{131}.

It is apparent that the resin sponge uptake of T_3-I^{131} is influenced by factors other than those contained within the sera. Hence, the physical characteristics of the sponge, the volume of sponge used, the equilibration temperature, the duration of equilibration, and the pH of the buffer may produce significant changes in the absolute values obtained with the sponge.

REFERENCES

1. MITCHELL, M. L.: Resin uptake of radiothyroxine in sera from non-pregnant and pregnant women, *J. Clin. Endocrinol. & Metab.* **18**: 1437, 1958.
2. MITCHELL, M. L., and O'ROURKE, M. E.: Differential resin binding of thyroxine in serum, *Clin. Res. Proc.* **7**: 241, 1959.
3. MITCHELL, M. L.; WHITEHEAD, W. O., and O'ROUKRE, M. E.: Differential adsorption of hormones from serum: a new procedure, *J. Lab. & Clin. Med.* **52**: 930, 1958.
4. MITCHELL, M. L.; WHITEHEAD, W. O., and O'ROURKE, M. E.: Differential resin binding of insulin in serum, *Endocrinology* **65**: 322, 1959.
5. GORDON, A. H.; GROSS, J.; O'CONNOR, D., and PITT-RIVERS, R.: Nature of the circulating thyroid hormone-plasma protein complex, *Nature* **169**: 19, 1952.
6. INGBAR, S. H.: Pre-albumin: a thyroxine-binding protein of human plasma, *Endocrinology* **63**: 256, 1958.
7. RICHARDS, J. B.; DOWLING, J. T., and INGBAR, S. H.: Alterations in the plasma transport of thyroxine in sick patients and their relation to the abnormality in Graves' disease, *J. Clin. Invest.* **38**: 1035, 1959.

8. HAMOLSKY, M. W.; STEIN, M., and FREEDBERG, A. S.: The thyroid hormone-plasma protein complex in man. II. A new *in vitro* method for study of "uptake" of labelled hormonal components by human erythrocytes, *J. Clin. Endocrinol. & Metab.* **17**: 33, 1957.
9. MITCHELL, M. L.; O'ROURKE, M. E., and HARDEN, A. B.: Unpublished observations.
10. ROBBINS, L. R.: Experience with *in vitro* erythrocyte uptake of I^{131}-labeled-*l*-triiodothyronine in a routine clinical laboratory, *J. Clin. Endocrinol. & Metab.* **19**: 1292, 1959.
11. SHARPE, A. R., JR.: *In vitro* erythrocyte uptake of I^{131}-triiodothyronine as a test of thyroid function, *Clin. Res. Proc.* **8**: 85, 1960.

SIGNIFICANCE OF THE CONCENTRATION OF NONPROTEIN-BOUND PLASMA CORTISOL IN NORMAL SUBJECTS, CUSHING'S SYNDROME, PREGNANCY, AND DURING ESTROGEN THERAPY*†

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ABSTRACT

The concentrations of total 17-hydroxycorticosteroid (17-OH-CS) and protein-bound 17-OH-CS were determined in plasma from various normal and abnormal subjects. The concentration of nonprotein-bound 17-OH-CS was then calculated by subtraction of the protein-bound value from the total 17-OH-CS value. Elevated plasma total 17-OH-CS concentration in Cushing's syndrome was shown to be due entirely to an increase in the nonprotein-bound 17-OH-CS fraction. Conversely, the elevated plasma total 17-OH-CS concentration in estrogen-treated males without signs of Cushing's syndrome was shown to be due entirely to an increase in the protein-bound 17-OH-CS. In women in the third trimester of pregnancy (with signs of mild adrenocortical hyperfunction) the levels of both protein-bound and nonprotein-bound plasma 17-OH-CS were increased. It is suggested that the nonprotein-bound plasma 17-OH-CS fraction is the immediately active form of cortisol. It probably has high activity because it is able to diffuse readily across capillaries and cell membranes.

IT IS known that cortisol is the major steroid with significant glucocorticoid activity secreted by the normal human adrenal cortex. Measurements of plasma cortisol concentration have invariably been made following extraction with an organic solvent such as chloroform or methy-

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lene chloride. This procedure denatures the plasma proteins, and extracts virtually all of the protein-bound and nonprotein-bound cortisol. Recently the binding of cortisol by plasma protein has been studied intensively. Daughaday (1, 2), utilizing radioactive 4-C^{14}-cortisol exchange methods *in vitro*, found that at normal plasma concentrations, cortisol was bound firmly to a protein or proteins associated with Cohn fraction IV-4, or more specifically, one of the alpha globulins. At higher total plasma cortisol concentrations (usually above 30 μg. per 100 ml.), the additional cortisol was said to be bound by albumin. More recently Slaunwhite and Sandberg (3, 4) modified the cortisol 4-C^{14} exchange technique, and also found cortisol to be bound to an alpha globulin. Moreover, they found evidence of an increased binding capacity in the third trimester of pregnancy—a period known to be associated with elevated plasma cortisol levels (5). This finding conflicted with earlier work by Daughaday in which no evidence of increased binding was observed in the third trimester of pregnancy (6).

Relatively sparse data are available on protein binding of cortisol *in vivo*. Peterson and collaborators (7) and Mills and associates (8) developed techniques utilizing ultrafiltration at 37° C., and isotope dilution techniques with paper chromatography, to study normal and estrogen-treated subjects. *In vivo* studies would appear to be of importance, since the liver and perhaps other organs are known to play a major role in binding various substances to protein. For instance, copper added to serum *in vitro* becomes bound exclusively to serum albumin, whereas *in vivo* it initially becomes bound to serum albumin but after two hours is found mainly in the $alpha_2$ globulin (ceruloplasmin) fraction (9). Estrogen added to plasma will not become bound to albumin *in vitro* unless minced liver homogenates are added (10).

The purpose of the present study was to examine the binding of plasma cortisol, as present *in vivo*, by chemical techniques without the addition of exogenous cortisol.

MATERIALS AND METHODS

Studies were performed on 11 normal male subjects, 4 patients with Cushing's syndrome due to bilateral adrenal hyperplasia, 1 patient with typical Cushing's syndrome secondary to carcinoma of the lung, 16 females in the third trimester of pregnancy, and 30 males with carcinoma of the prostate receiving 5 mg. of diethylstilbestrol orally for more than two weeks. Two of these 30 men were also studied after a standard four-hour infusion of corticotropin (ACTH).

A 50-ml. sample of blood was drawn from each subject. Half of the sample was separated as plasma, subjected to carbon tetrachloride and petroleum ether wash, and analyzed for 17-hydroxycorticosteroids (17-OH-CS) by the Peterson *et al.* modification (11) of the method of Silber and Porter (12). The duplicate half of the blood sample was separated as serum and divided into its various protein components at 5° C. and pH 8.6 in Veronal buffer by a previously reported modification (13) of the starch block

electrophoresis method of Kunkel and Slater (14). The starch block was cut into 1.5-cm. strips, and the starch separated from the protein by washing with 50 ml. of physiologic salt solution through a sintered glass filter which retained the larger starch particles. A 1-ml. portion of each eluate was analyzed for protein by a tyrosine method (15). These values served as reference points for the corresponding electrophoretic curves. The remaining 49-ml. portion of each fraction was then concentrated to 2 ml. by suction dialysis through a collodion membrane and analyzed for 17-OH-CS. This step removes any nonprotein-bound steroid which may have undergone migration in the electrical field. It also reduces the sample to a convenient volume.

The total amount of 17-OH-CS recovered from the protein fractions of each serum sample was used as the plasma protein-bound 17-OH-CS value. Subtraction of this value from the total plasma 17-OH-CS level gave the nonprotein-bound 17-OH-CS level of the plasma. Blank values were determined on portions of the starch block not exposed to serum; they were extremely low in all instances.

Twenty-eight of the estrogen-treated males were studied by a simplified technique in which the plasma protein-bound 17-OH-CS concentration was obtained by subjecting the duplicate serum sample directly to suction dialysis at 5° C., a 25-ml. saline wash, and analysis of the protein concentrate for 17-OH-CS without separation on the starch block. Nonprotein-bound 17-OH-CS concentration was estimated by subtraction of protein-bound 17-OH-CS from total plasma 17-OH-CS as before. Values obtained in this manner were identical to those obtained by the longer procedure. Therefore, the results were considered to be interchangeable between these two almost identical methods of measurement of the nonprotein-bound 17-OH-CS plasma level in estrogen-treated patients.

It has been demonstrated that the plasma 17-OH-CS content is almost entirely cortisol in normal subjects (15) and in estrogen-treated males (16). In the interpretation of the results of this study it is assumed that the 17-OH-CS values represent mainly cortisol.

RESULTS

Figure 1 shows the results obtained in 11 normal male subjects. In every instance the alpha$_1$ globulin contained more 17-OH-CS than did any other fraction.[1] This finding was accentuated when the results were calculated per gram of protein in the fraction. The mean total plasma 17-OH-CS value for this group was 17.2 μg. per 100 ml. of plasma. The mean con-

[1] These 17-OH-CS were further identified as follows: Four 12-ml. samples of plasma from normal subjects were subjected to starch block electrophoresis. The areas of the block representing albumin, and alpha$_1$, alpha$_2$, beta, and gamma globulin were separated and combined, the starch removed as described previously, and each dissolved in water. The aqueous solutions were washed with carbon tetrachloride and petroleum ether, and the steroids extracted with methylene chloride. These extracts were dried separately and studied with paper chromatography, utilizing isotope dilution methods (17) to correct for losses. The following results were obtained:

1. Cortisol was present in the alpha$_1$ globulin fraction and was identified by: (a) migration rates in Bush B5 (18) and E_2B (19) systems identical to those of purified cortisol and 4-C^{14} cortisol standards; (b) ultraviolet absorbance maximum at 241 mμ in methanol; (c) positive staining reaction with alkaline blue tetrazolium; (d) fluoresence

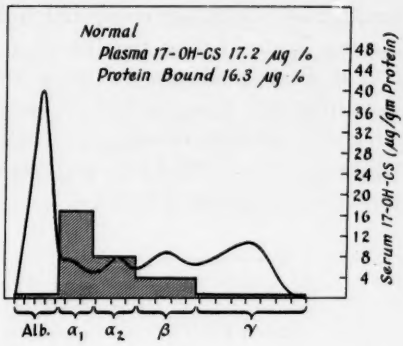

FIG. 1. Mean concentration and distribution of serum 17-OH-CS in 11 normal subjects. The mean plasma total 17-OH-CS concentration averaged 17.2 μg. per 100 ml., of which 16.3 μg. per 100 ml. was protein bound.

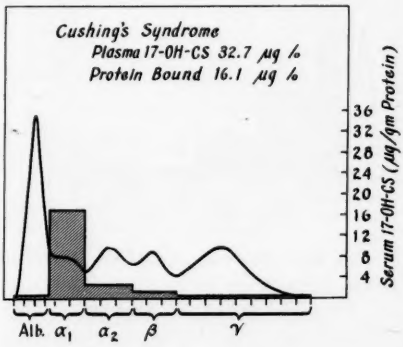

FIG. 2. Mean concentration and distribution of serum 17-OH-CS in 4 patients with Cushing's syndrome due to bilateral adrenal hyperplasia. The mean plasma total 17-OH-CS concentration was 32.7 μg. per 100 ml., of which 16.1 μg. per 100 ml. was protein bound. Concentration of protein-bound steroid was not significantly different from that in the normal subjects.

centration of protein-bound 17-OH-CS was 16.3 μg. per 100 ml. of plasma. Thus 0.9 μg. per 100 ml. was the mean nonprotein-bound plasma 17-OH-CS concentration, with a range of −5.3 μg. per 100 ml. to +8.4 μg. per 100 ml. This finding is in agreement with those of Daughaday (1) and Slaunwhite and Sandberg (3) in normal subjects utilizing 4-C^{14} exchange methods *in vitro*, and with those of Mills and collaborators (8). The mean concentration was 17 μg. of 17-OH-CS per gram of $alpha_1$ globulin.

Figure 2 illustrates the corresponding 17-OH-CS values in 4 subjects with adrenal hyperfunction due to bilateral hyperplasia. In spite of an elevated total plasma 17-OH-CS concentration of 32.7 μg. per 100 ml., the

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emission spectrum in sulfuric-acid ethanol identical to that of cortisol.

2. The presence of 1.63 μg. of cortisol in the $alpha_1$ globulin fraction representing 45 ml. of human plasma was determined by isotope dilution.

3. No cortisol was detectable in any of the other protein fraction extracts by the methods used. This indicates that less than 1 μg. was present in each of these fractions.

4. No steroids other than cortisol were detected by these methods.

amount of 17-OH-CS recovered from protein was 16.1 μg. per 100 ml. of plasma. Thus, 16.6 μg. per 100 ml. (range +13.7 to +19.2) was nonprotein bound. Statistical analysis using the *t* test for unpaired variates showed this difference from normal to be significant, with $P < .01$. Again, almost all of the protein binding of 17-OH-CS occurred in the alpha$_1$ globulin fraction, and 17-OH-CS were present in a normal concentration of 17 μg. per gram of alpha$_1$ globulin.

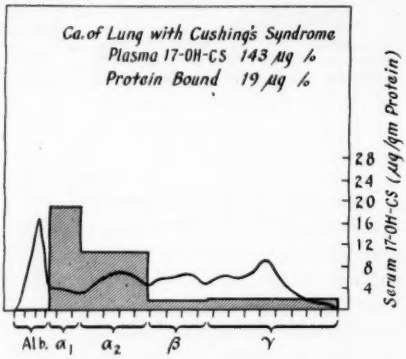

FIG. 3. Concentration and distribution of serum 17-OH-CS in a patient with a typical Cushing's syndrome which was secondary to a carcinoma of the lung. The plasma total 17-OH-CS concentration was 143 μg. per 100 ml., of which only 19 μg. per 100 ml. was protein bound.

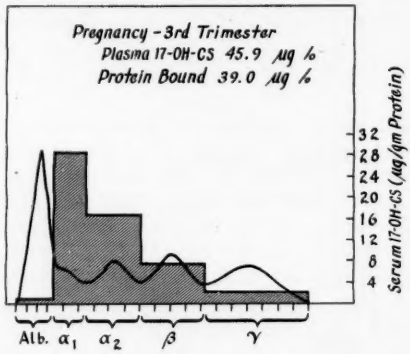

FIG. 4. Increase in 17-OH-CS bound to alpha$_1$ globulin in 16 pregnant females in the third trimester of pregnancy. The plasma total 17-OH-CS concentration was 45.9 μg. per 100 ml., of which 39.0 μg. per 100 ml. was protein bound.

Figure 3 shows the results obtained in a subject with bilateral adrenal hyperplasia secondary to carcinoma of the lung. The plasma level of total 17-OH-CS was 143 μg. per 100 ml., but the protein-bound 17-OH-CS level was normal (19 μg. per 100 ml.). Therefore, of the 17-OH-CS, 124 μg. per 100 ml. of plasma was not protein bound. This form of secondary adrenal hyperfunction was thus associated with a large increase in the concentration of nonprotein-bound 17-OH-CS. It would perhaps be unwise to consider all of this 17-OH-CS elevation as being due to cortisol, without further proof, although the patient had a severe hypokaliemic alkalosis, diabetes, hypertension, plethoric facies, and all the findings of acute severe Cushing's syndrome.

Figure 4 shows the data on 16 pregnant females in the third trimester of pregnancy. The mean plasma level of total 17-OH-CS was 45.9 μg. per 100 ml.—a value higher than the mean level in the adrenal hyperfunction

group. However, in contrast to the patients with adrenal hyperfunction, a much higher percentage of the plasma 17-OH-CS was protein-bound (39.0 μg. per 100 ml.). The fact that 35 μg. of 17-OH-CS were found per gram of alpha$_1$ globulin constituted further evidence of an increase in the protein binding of cortisol. The mean concentration of unbound 17-OH-CS was 7.3 μg. per 100 ml. of plasma (range, −2.2 to +13.5)—also significantly elevated ($P < .05$) from normal in spite of the increased protein binding.

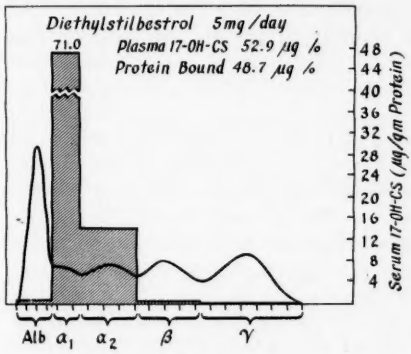

FIG. 5. Rise in plasma total 17-OH-CS concentration after estrogen therapy in 30 males. All of this elevation was accounted for by the rise in plasma protein-bound 17-OH-CS concentration. The electrophoretic distribution is typical (based on data for 2 of the 30 patients studied).

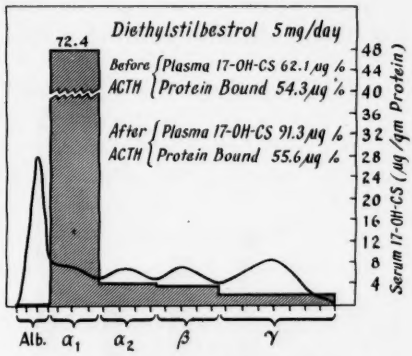

FIG. 6. Effect of a four-hour infusion of 25 units of ACTH upon the plasma 17-OH-CS concentration in the 2 males for whom the electrophoretic distribution is shown in Figure 5. No change in the level of protein-bound 17-OH-CS was detected in spite of the elevation of the total 17-OH-CS concentration.

When a daily dose of 5 mg. of diethylstilbestrol was administered to each of 30 males with carcinoma of the prostate (Fig. 5), there was a rise in plasma total 17-OH-CS concentration to a value above that found in Cushing's syndrome and the third trimester of pregnancy. The mean plasma total 17-OH-CS level was 52.9 μg. per 100 ml., of which 48.7 μg. per 100 ml. was protein bound. The mean concentration of nonprotein-bound 17-OH-CS was 4.2 μg. per 100 ml. (range, −10.1 to +15.3)—not significantly different from normal. The amount of 17-OH-CS bound to alpha$_1$ globulin was increased to 71.9 μg. per gram. After a four-hour infusion of ACTH the plasma level of total 17-OH-CS rose to 91 μg. per 100 ml. without a significant change in the protein-bound 17-OH-CS concentration in the alpha$_1$ globulin fraction (Fig. 6). Thus the rise in plasma

17-OH-CS concentration after administration of ACTH was due entirely to an increase in the nonprotein-bound fraction. In 7 normal subjects after infusion of ACTH, there was a rise only in the nonprotein-bound plasma concentration—mean rise, 27 μg. per 100 ml.; range, 20 to 36 μg. per 100 ml.

In Figure 7 the foregoing findings are summarized.

In each group of subjects a variable amount of Porter-Silber chromogen was found in the alpha$_2$ globulin protein. However, this was always less than the amount found in alpha$_1$ globulin in spite of the fact that more protein was consistently present in alpha$_2$ globulin. Most of the cortisol in the alpha$_2$ globulin fraction could be accounted for by curving of the

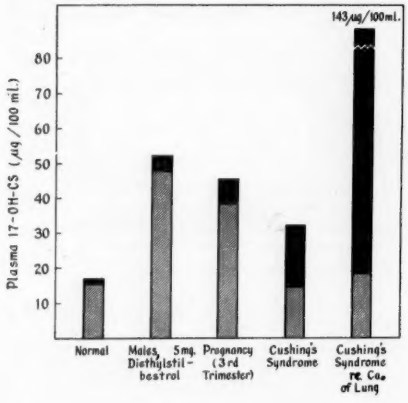

FIG. 7. Graphic summary of data on all patients. The mean concentration of nonprotein-bound plasma 17-OH-CS was 0.9 μg. per 100 ml. in normal subjects, 16.6 μg. per 100 ml. in Cushing's syndrome, 7.3 μg. per 100 ml. in the third trimester of pregnancy, 4.2 μg. per 100 ml. during estrogen therapy in males, and 35.7 μg. per 100 ml. after administration of ACTH in the same estrogen-treated males. The elevation in Cushing's syndrome and in pregnancy was significant.

electrophoretic front in some of the runs, which caused some alpha$_1$ globulin to be included in the alpha$_2$ globulin. In individual instances in which the electrophoretic front formed a straight line, no significant amount of Porter-Silber chromogen was found in the alpha$_2$ globulin. In every case studied, the Porter-Silber chromogen in the alpha$_1$ globulin formed a peak at 410 mμ, whereas this was not true of the other fractions. Nevertheless the possibility exists that a small amount of cortisol may be bound by either alpha$_2$ globulin or beta globulin and that this binding is too small in extent to be detected by the present procedure.

DISCUSSION

The clinician needs a clearer chemical definition of Cushing's syndrome. Cases of this syndrome are known to occur occasionally in the presence of normal or high-normal plasma 17-OH-CS concentrations. In addition, during administration of estrogen the relationship between the clinical status of the patient and the plasma total 17-OH-CS concentration is not only poor but misleading. The data presented here show a good correlation

between the clinical evaluation of adrenal functional status and the concentration of plasma nonprotein-bound 17-OH-CS in normal subjects, Cushing's syndrome, pregnancy, and during administration of estrogen.

Recently we reported on the absence of a diurnal variation of plasma 17-OH-CS levels in subjects with bilateral adrenal hyperplasia, and emphasized the importance of the resultant elevated nocturnal 17-OH-CS levels in causing clinical symptoms (20). It is possible that the nocturnal concentration of nonprotein-bound 17-OH-CS in plasma will prove to be an even more significant measurement in Cushing's syndrome. Studies are in progress.

Evidence has been presented in this study that in the third trimester of pregnancy there is increased protein binding of cortisol in plasma and also an increased concentration of nonprotein-bound cortisol. The increased binding involves both the absolute amount of 17-OH-CS bound to the alpha$_1$ globulin fraction and the amount of 17-OH-CS bound per gram of this globulin. The pregnant women in this study did not manifest profound clinical evidence of a Cushing's syndrome. However, many of them exhibited mild symptoms of adrenal hyperfunction, including purplish striae, glucosuria, hypertension, or increased fragility of cutaneous blood vessels. These mild symptoms seem best explained by the equally mild but significant increase in the concentration of nonprotein-bound plasma 17-OH-CS. No increase of the total alpha$_1$ globulin itself was detected in the pregnant women or in the estrogen-treated males.

However, the cortisol-binding protein (transcortin) is undoubtedly a trace protein, and constitutes only a small fraction of the total alpha$_1$ globulins. Therefore, this study cannot differentiate between increased binding due to an increase in concentration of a specific alpha$_1$ globulin and that due to increased activity of the globulin already available.

We have found that the *in vitro* addition of estrogens to plasma from normal subjects does not significantly increase the cortisol-binding capacity of the plasma upon incubation at 37° C. for twenty-four hours, even with simultaneous *in vitro* cortisol loading. *In vivo*, however, the administration of large amounts of estrogen to males for three to seven days results in an increase of cortisol binding which exceeds that in the pregnancy group. The mechanism of this increase of cortisol binding by alpha$_1$ globulin under conditions of estrogen excess will probably not be further clarified until direct isolation and study of the alpha$_1$ globulin subfraction involved has been achieved.

REFERENCES

1. Daughaday, W. H.: Binding of corticosteroids by plasma proteins. III. The binding of corticosteroid and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis, *J. Clin. Invest.* **37**: 511, 1958.

2. Daughaday, W. H.: Binding of corticosteroids by plasma proteins. IV. The electrophoretic demonstration of corticosteroid binding globulin, *J. Clin. Invest.* **37**: 519, 1958.
3. Slaunwhite, W. R., Jr., and Sandberg, A. A.: Transcortin: a corticosteroid-binding protein of plasma, *J. Clin. Invest.* **38**: 384, 1959.
4. Sandberg, A. A., and Slaunwhite, W. R., Jr.: Transcortin: a corticosteroid-binding protein of plasma. II. Levels in various conditions and the effects of estrogens, *J. Clin. Invest.* **38**: 1290, 1959.
5. Gemzell, C. A.: Variations in plasma levels of 17-hydroxycorticosteroids in mother and infant following parturition, *Acta endocrinol.* **17**: 100, 1954.
6. Daughaday, W. H.: Binding of corticosteroids by plasma proteins. V. Corticosteroid-binding globulin in normal human beings and in certain disease states, *A.M.A. Arch. Int. Med.* **101**: 286, 1958.
7. Peterson, R. E.; Nokes, G.; Chen, P. S., Jr., and Black, R. L.: Estrogens and adrenocortical function in man, *J. Clin. Endocrinol. & Metab.* **20**: 495, 1960.
8. Mills, I. H.; Schedl, H. P.; Chen, P. S., Jr., and Bartter, F. C.: The effect of estrogen administration on the metabolism and protein binding of hydrocortisone, *J. Clin. Endocrinol. & Metab.* **20**: 515, 1960.
9. Bearn, A. G.: Wilson's disease. An inborn error of metabolism with multiple manifestations, *Am. J. Med.* **22**: 747, 1957.
10. Szego, C. M., and Roberts, S.: Hepatic intervention in the binding of estrogen to rat serum albumin in vitro, *J. Biol. Chem.* **221**: 619, 1956.
11. Peterson, R. E.; Karber, A., and Guerra, S. L.: Evaluation of the Silber-Porter procedure for the determination of plasma hydrocortisone, *Analyt. Chem.* **29**: 144, 1957.
12. Silber, R. H., and Porter, C. C.: The determination of 17,21-dihydroxy-20-ketosteroids in urine and plasma, *J. Biol. Chem.* **210**: 923, 1954.
13. Zinneman, H. H.; Glenchur, H., and Hall, W. H.: The nature of blocking antibodies in human brucellosis, *J. Immunol.* **83**: 206, 1959.
14. Kunkel, H., and Slater, R. J.: Zone electrophoresis in a starch supporting medium, *Proc. Soc. Exper. Biol. & Med.* **80**: 42, 1952.
15. Greenberg, D. M.: The colorimetric determination of the serum proteins, *J. Biol. Chem.* **82**: 545, 1929.
16. Peterson, R. E.; Wyngaarden, J. B.; Guerra, S. L.; Brodie, B. B., and Bunim, J. J.: The physiological disposition and metabolic fate of hydrocortisone in man, *J. Clin. Invest.* **34**: 1779, 1955.
17. Peterson, R. E.; Kliman, B., and Bollier, M. E.: A physical method for determination of steroids in biological extracts. Abstracts of the 131st Meeting of the American Chemical Society, Miami, Florida, April 7–12, 1957 (59C).
18. Bush, I. E.: Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues, *Biochem. J.* **50**: 370, 1951.
19. Eberlein, W. R., and Bongiovanni, A. M.: New solvent systems for the resolution of corticosteroids by paper chromatography, *Arch. Biochem. & Biophys.* **59**: 90, 1955.
20. Doe, R. P.; Vennes, J. A., and Flink, E. B.: Diurnal variation of 17-hydroxycorticosteroids, sodium, potassium, magnesium and creatinine in normal subjects and in cases of treated adrenal insufficiency and Cushing's syndrome, *J. Clin. Endocrinol. & Metab.* **20**: 253, 1960.

ADRENAL FUNCTION IN A PREGNANT BILATERALLY ADRENALECTOMIZED WOMAN*

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ABSTRACT

A case is presented of successful pregnancy in a patient who had undergone bilateral adrenalectomy for Cushing's syndrome. Studies of plasma and urinary steroids were performed before and after treatment during pregnancy and post partum. The observations suggest that the increased urinary excretion of aldosterone and the increased adrenal "responsiveness" to corticotropin seen in the normal pregnant subject require the presence of an intact adrenal gland, and are not mediated through the placenta. The increase in the half-life of hydrocortisone in pregnancy is dependent upon extra-adrenal factors.

SUCCESSFUL completion of pregnancy is being reported more frequently in patients with Addison's disease treated by hormonal replacement therapy (1–11), but few patients (12–14) who have undergone bilateral adrenalectomy for Cushing's syndrome have had subsequent successful pregnancies. This report presents studies on one such patient during and after gestation.

CASE REPORT

L. M. was a 26-year-old female, one of identical twins. Her twin had no history of illness. The patient had developed normally. The menarche occurred at age 11½ years. Beginning in 1951, at age 19 years, she experienced episodes of amenorrhea and oligomenorrhea. From 1952 to 1955 (age 20–23 years) there was total amenorrhea except when she was given cyclical therapy with estrogen and progesterone. In 1952 she noted the onset of facial hirsutism, acne and weakness. By 1955 violaceous abdominal striae, easy bruisability, a buffalo hump, truncal obesity and thinning of the scalp hair had appeared. The blood pressure readings, which had previously been normal, ranged as high as 200 mm. Hg systolic.

The first hospital admission was in April 1955. Physical examination revealed a buffalo hump, plethora, moon facies, slight exophthalmos, slight facial hirsutism, multiple ecchymoses and a normal female escutcheon. No abnormality was noted in the

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pelvis or external genitalia. The blood pressure was 185/120 mm. Hg. Laboratory studies demonstrated moderately impaired glucose tolerance (fasting blood sugar level 88 mg. per 100 ml.; half-hour, 141 mg.; one hour, 202 mg.; two hours, 192 mg.; and three hours, 129 mg.). A complete blood count and urinalysis findings were within normal limits. The concentration of serum sodium was 147 mEq. per liter; potassium, 4.4 mEq. per liter; calcium, 11 mg. per 100 ml.; and phosphorus, 3.2 mg. per 100 ml. The urinary gonadotropin titer was positive at 5 mouse uterine units but negative at 50 units. A vaginal smear showed evidence of estrogen deficiency. Roentgenograms of the skull and spine showed a normal sella turcica and no evidence of demineralization. The intra-

TABLE 1. ADRENAL FUNCTION IN WOMAN ADRENALECTOMIZED FOR CUSHING'S DISEASE (data prior to pregnancy)

Date	Therapy	Urinary steroids (mg./24 hrs.)			Plasma free 17-OH-corticoids (Porter-Silber) (μg./100 ml.)	
		Formaldehydogenic corticoids	17-OH-corticoids (Porter-Silber)	Neutral 17-ketosteroids	Basal	After ACTH
1955						
April	Untreated	6.0		23.1		
	ACTH-gel, 80 U. for 3 days	17.7		35.0		
May	X-rays, 3000 r. to pituitary	—		—		
August	X-rays, 1000 r. to pituitary	—		—		
Sept. 20	—	2.5		14.8		
1956						
Jan. 20	*Rt. adrenalectomy*	—		—		
Apr. 4	—	3.9		12.2	19	—
May	—	1.4		—	25	58
July	X-rays, 4000 r. to pituitary	—		—	—	—
August	—	2.8	10.6	—	33	68
Aug. 14–21	Prednisone, 40 mg. q.d.	—	—	—	—	—
Aug. 21	—	—	—	—	30	48
October	—	—	13.6	—	—	—
1957						
Jan. 25	*Lt. adrenalectomy.* Maintained with 37.5 mg. cortisone q.d. and 75 mg. DOC-tma. q.mo.	—	—	—	—	—
June	↓	—	9.5	8.5	—	—

venous pyelogram was normal. The results of studies of adrenal function are presented in Table 1.

A diagnosis of Cushing's syndrome was made, and the patient was given a course of pituitary x-irradiation in a dosage of 4000 r delivered to the gland. This failed to induce any change in her symptoms or physical appearance. Laboratory evidence of adrenocortical hyperfunction persisted (September 20, 1955—Table 1).

In January 1956, she was readmitted for a right adrenalectomy. The findings on physical examination and laboratory studies were unchanged from those noted during the first admission. Roentgenograms of the spine at this time, however, showed evidence of generalized demineralization. Right adrenalectomy was performed uneventfully on January 20, 1956. The adrenal weighed 10 grams and was grossly normal. The pathologic report was an adrenal gland without significant change except for extensive recent

hemorrhage, chiefly in the medulla, which was probably operative in origin. The postoperative course was benign. The blood pressure ranged from 140/70–140/90 mm. Hg.

Following operation there was some improvement in the patient's plethora and hirsutism. However, the moon facies, amenorrhea, easy bruisability and weakness persisted. The urinary excretion of formaldehydogenic corticoids remained elevated (April 4, 1956—Table 1). The vaginal smear still showed evidence of estrogen deficiency. The urinary gonadotropin titer was positive at 5 and 50 mouse uterine units, but negative at 100 units. In July 1956, she was again given a course of pituitary irradiation (4000 r delivered to the gland) without subsequent clinical improvement. There was no suppression of the plasma corticoid response to administration of adrenocorticotropin (ACTH) following a week of therapy with prednisone (Table 1).

The patient was hospitalized for the third time in January 1957. The findings on physical examination and laboratory studies were essentially unaltered. Left adrenalectomy was performed. The adrenal weighed 8 grams and was normal on section except for the presence of several tiny cortical adenomata. On discharge, she was receiving a maintenance dosage of 37.5 mg. of cortisone per day, to which desoxycorticosterone trimethylacetate (50 mg. intramuscularly once a month) was added one month postoperatively. The latter dosage was subsequently increased to 75 mg. because of her persistent complaints of weakness. The hirsutism, moon facies and easy bruisability gradually disappeared over the following four-month period. The blood pressure was maintained at 130/80 mm. Hg.

Spontaneous menstrual periods occurred two and three months postoperatively. Basal body temperature records failed to indicate ovulation. Vaginal smears at this time showed a good estrogenic effect. There were no further spontaneous menstrual periods for four months, until one was induced (August 1957) following an injection of 17α-hydroxyprogesterone caproate. On September 20, 1957, a spontaneous menstrual period occurred. Following this the basal body temperature chart showed evidence of ovulation. There was no drop in temperature prior to the expected date of the next menses, and no further menses occurred. Results of a pregnancy test performed on November 6, 1957, were positive.

The antepartum course was uneventful. Maintenance therapy remained unchanged until the sixth month of gestation. At this time, cortisone (37.5 mg. daily) was continued, but 9α-fluorohydrocortisone (0.2 mg. daily) was substituted for the desoxycorticosterone trimethylacetate. On July 5, 1958, five hours after the beginning of labor, a healthy male infant weighing 5 pounds 13 ounces was delivered by low forceps. During labor the patient received 50 mg. of cortisone acetate intramuscularly and a slow intravenous drip of 5 per cent glucose containing 50 mg. of hydrocortisone hemisuccinate. The day following delivery, the usual medication was resumed.

Thus far, eighteen months post partum, the patient and child are doing well, save for the development of progressive ovarian insufficiency in the mother. This is indicated by a rising urinary gonadotropin titer (positive at 10 mouse uterine units eight months post partum, and positive at 100 mouse uterine units eighteen months post partum), and the failure of 17α-hydroxyprogesterone caproate to induce uterine bleeding when administered at intervals during the tenth to eighteenth months post partum whereas it induced bleeding when administered during the sixth to eight months post partum. Roentgenograms of the skull show no enlargement of the sella turcica. Thyroid function is normal. The short vagina is of less than normal capacity with a thin mucosa, and the uterus is very small.

CLINICAL STUDIES

The tests of adrenal function performed during and subsequent to the pregnancy are shown in Table 2. Urinary neutral 17-ketosteroids were assayed by the method of Holtorff and Koch (15). Formaldehydogenic corticoids were measured by the method of Corcoran and Page (16).

TABLE 2. DATA DURING PREGNANCY AND POST PARTUM

Stage of gestation and date in 1958	Therapy	Urinary steroids (mg./24 hrs.)		Plasma free 17-OH-corticoids (Porter-Silber) (μg./100 ml.)	
		17-OH-corticoids (Porter-Silber)	Neutral 17-ketosteroids	Basal	After ACTH
Second trimester					
Week 15, Jan. 22	Cortisone, 37.5 mg. q.d., and DOCtma.,* 75 mg. q. mo.	—	—	9.0†	21.2
Week 16, Feb. 4	↓	11.1	11.5	—	—
Week 17, Feb. 19	↓	11.6	8.4	15.6†	19.4
Week 22, Apr. 22	↓	10.0	9.8	—	—
Apr. 23	0	13.0	9.1	—	—
Apr. 24	0	8.3	8.6	—	—
Apr. 25	0	8.3	8.4	11.8	17.0
Apr. 26	Cortisone 37.5 mg. q.d., and 9 α-fl.F,** 0.2 mg. q.d.	10.7	13.0	—	—
Third trimester	↓				
Week 33, June 17	Cortisone, 37.5 mg. q.d.	12.0	11.9	—	—
June 18	0	—	—	—	—
June 19	0	—	—	—	—
June 20	0	2.7	5.4	—	—
June 21	0	—	—	—	—
June 22	0	—	—	—	—
June 23	0	4.2	7.4	11.0	18.5
June 24	Cortisone, 37.5 mg. q.d., and 9α-fl.F, 0.2 mg. q.d.	—	—	—	—
Labor	↓				
July 5, 12:30 a.m.	↓	—	—	17.5‡	—
3:30 a.m.	Cortisone, 50 mg. i.m.	—	—	—	—
	Hydrocortisone, 50 mg. i.v.	—	—	—	—
5:30 a.m.		—	—	20.5§	—
Post partum					
July 6	Cortisone, 37.5 mg. q.d., and 9α-fl. F, 0.2 mg. q.d.	—	—	29.4‖	—
July 8	↓	21.8	7.8	39.4†	—
July 10	↓	15.2	5.5	—	—
July 11	↓	10.7	11.6	—	—
Six weeks post partum	↓				
Aug. 24–27	0	—	—	—	—
Aug. 27	0	0.0	2.26	19.0	—

* Desoxycorticosterone trimethylacetate.
** 9α-Fluorohydrocortisone.
† Blood drawn three hours after daily dose of cortisone.
‡ Ten hours after last maintenance dose of 12.5 mg. of cortisone and 0.1 mg. of 9α-fl. F.
§ Two hours after 50 mg. of cortisone i.m. and 50 mg. of hydrocortisone i.v. The concentration in umbilical blood at this time was 10.3 μg. per 100 ml.
‖ Prior to reinstitution of therapy.

Plasma and urinary 17-hydroxycorticoids were measured by a modification of the Silber-Porter method (17). Aldosterone secretion rates were kindly measured by Dr. Stanley Ulick (18).

In the second trimester of pregnancy there was no significant response, or at best a markedly impaired response of the plasma 17-hydroxycorticoids to the administration of ACTH, regardless of whether or not the patient was receiving replacement therapy. There was a slight drop in urinary 17-hydroxycorticoid excretion after discontinuance of replacement therapy for three days, with no change in the excretion of neutral 17-ketosteroids.

A similar study was performed in the third trimester of pregnancy when replacement therapy was discontinued for a period of one week. No adverse clinical effect was noted save for the appearance of slight labial herpes. The level of plasma 17-hydroxycorticoids remained unaltered, and there was no significant response to the administration of ACTH. There was a drop in the urinary excretion of 17-hydroxycorticoids and neutral 17-ketosteroids. The half-life of hydrocortisone, determined by measuring the concentration of plasma free 17-hydroxycorticoids following an infusion of 100 mg. of hydrocortisone hemisuccinate, was 240 minutes (for comparison, the half-life is 90 minutes in the normal non-

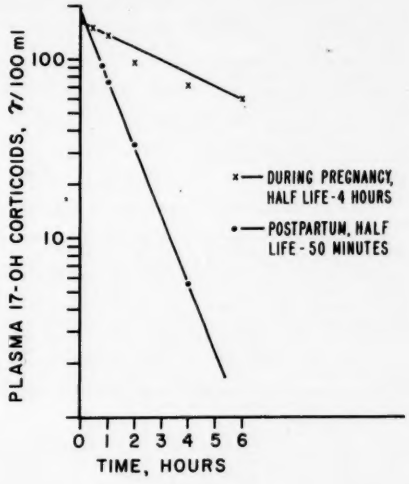
PLASMA 17-OH CORTICOIDS, γ/100 ml
100
10
0 1 2 3 4 5 6
TIME, HOURS
× — DURING PREGNANCY, HALF LIFE - 4 HOURS
• — POSTPARTUM, HALF LIFE - 50 MINUTES

FIG. 1. Half-life of plasma free 17-hydroxycorticoids, during and after pregnancy.

pregnant woman and 140–180 minutes in the normal pregnant woman (19)). The aldosterone secretion rate was 25 μg. per twenty-four hours (for comparison, secretion in the normal nonpregnant woman is greater than 200 μg. per twenty-four hours).

Six weeks post partum, replacement therapy was again discontinued for a period of only four days. This was associated with the development of an upper respiratory infection, hypotension, and hemoconcentration. During this period 17-hydroxycorticoids disappeared from the urine and neutral 17-ketosteroid excretion was diminished. The plasma free 17-hydroxycorticoid level was normal. The half-life of hydrocortisone was 50 minutes (Fig. 1). Aldosterone secretion was unchanged from that observed during pregnancy.

Table 3 shows the results of fractionation studies of the 17-ketosteroids and cortisol metabolites during these periods. During pregnancy the values

for the isolated steroids were all low, particularly those of the C21 metabolites. Androsterone and etiocholanolone, although in small amounts, were definitely present. Post partum, there was a marked drop in the excretion of androsterone and etiocholanolone. Indeed, it is questionable whether these compounds were even present, but lack of material precluded further study.

Eighteen months post partum, the patient was studied for two separate seven-day periods during which she received 1.5 mg. of dexamethasone

TABLE 3. STEROIDS ISOLATED (MG./24 HRS.)*

Period	KS**	BT	A	E	OHA	OHE	11-OE	THE	THF	ATF
Second trimester of pregnancy										
Three days after cessation of steroid medication	6.6	6.4	0.5	0.5	0.3	0.4	0.6	0.5	0.8	0.3
Third trimester of pregnancy										
Seven days after cessation of steroid medication	10.7	7.6	0.3	0.5	0.3	0.3	1.4	0.8	0.6	tr
Six weeks post partum										
Three days after cessation of steroid medication	—	2.9	—	—	—	—	—	0.2	0.2	—
Three months post partum										
Maintenance steroid medication	4.6	9.0	0.06	0.01	—	—	—	—	—	—

* These steroid analyses were performed through the courtesy of Dr. T. F. Gallagher at the Sloan-Kettering Institute, New York City.

The area in the chromatogram of steroids from urine of the second and third trimesters, corresponding in mobility to androsterone, etiocholanolone and 11-ketoetiocholanolone, were combined and re-chromatographed on paper as the free steroids. The androsterone area was identified conclusively from the infrared spectrum; the etiocholanolone area was similar to the reference spectrum but showed additional absorption; the area corresponding to 11-ketoetiocholanolone could have contained this steroid, judging from the infrared spectrum, but there was so much additional contaminating material that no valid conclusion was possible by this criterion. These 3 fractions, each acetylated and re-chromatographed on paper, showed material staining with the Zimmermann reagent in the same area as the reference standards of the respective acetates. Tetrahydro E and tetrahydro F in these periods were identified only by the mobility of the free steroids and their acetates with respect to pure reference standards; the amounts available were too small for infrared analysis. The absolute values for the steroids isolated in these periods may be in error by as much as 50 per cent because of the limitations imposed by the small amount of steroid analyzed in the presence of considerable background material. The difference between the second and third trimester periods and the three-month postpartum period is beyond the error of the method.

** Nomenclature:

KS = Combined values for Zimmermann chromogens of the "ketonic" fraction from Ketodase hydrolysis and subsequent acid hydrolysis of urinary conjugates.

BT = Blue tetrazolium chromogens ("tetrahydrocortisone" equivalents) from the neutral ether-soluble steroid fraction after Ketodase hydrolysis.

A = Androsterone.
E = Etiocholanolone.
OHA = 11-Hydroxyandrosterone.
OHE = 11Hydroxyetiocholanolone.
11-OE = 11-Ketoetiocholanolone.
THE = Tetrahydrocortisone.
THE = Tetrahydrocortisol.
ATF = Allotetrahydrocortisol.

and 0.1 mg. of 9α-fluorohydrocortisone daily. At the end of these periods the urinary excretion of 17-hydroxycorticoids was 4.1 and 2.2 mg. per twenty-four hours, and neutral 17-ketosteroid excretion was 3.3 and 4.8 mg. per twenty-four hours. Androsterone and etiocholanolone were each present in a concentration of 0.7 mg. per twenty-four hours. During a third seven-day period, ACTH-gel (40 units intramuscularly daily) was added to the foregoing regimen. At the end of this period the urinary excretion of 17-hydroxycorticoids was 7.1 mg. per twenty-four hours, and the excretion of neutral 17-ketosteroids was 5.6 mg. per twenty-four hours.

DISCUSSION

The role of the adrenal in the physiologic and chemical changes occurring in normal pregnancy is not yet fully understood. Pregnancy prolongs the

life of adrenalectomized dogs (20, 21), and pregnant women are stated to have larger adrenals than normal women (22, 23).

Studies of adrenal function during the third trimester of normal pregnancy have demonstrated that there is an increase in the plasma level of free Porter-Silber reacting material (19, 22–24); an increase in the plasma level of hydrocortisone has been demonstrated chromatographically (25).

An enhanced response of the plasma free 17-hydroxycorticoids to the administration of ACTH in the third trimester has also been reported (26). The urinary excretion of 17-hydroxycorticoids during pregnancy, however, is usually within normal limits (19, 27, 28). Several explanations have been offered to account for this discrepancy. It has been shown that the administration of estrogen to normal subjects results in an increase in the basal plasma level of free 17-hydroxycorticoids, an increased response of the plasma free 17-hydroxycorticoids to the administration of ACTH (25) and a prolonged half-life of hydrocortisone (29). These observations are duplicates of the phenomena observed in the normal pregnant state (29). On the other hand, some investigators have suggested the placenta as the source of the elevated corticoids observed in pregnancy (14, 30–34).

An increased urinary excretion of aldosterone has been reported to occur during normal pregnancy (35, 36). Studies of the neutral 17-ketosteroids show that, although the plasma levels may fall (25, 37), there is no significant alteration in the urinary levels (23, 25). Fractionation studies demonstrate, however, a decrease in urinary androsterone and etiocholanolone (25, 38) and a rise in 11-ketoetiocholanolone and 11-OH-androsterone during pregnancy.

Studies in Addisonian or bilaterally adrenalectomized pregnant subjects provide a method for determining whether or not the placenta is the source of the increased quantities of corticoids in the plasma and of the aldosterone excreted in the urine in normal pregnancy. One pregnant woman with Addison's disease (11), deprived of hormonal therapy in weeks 31–33 of gestation, excreted markedly reduced amounts of 17-hydroxycorticoids without any clinical evidence of adrenocortical insufficiency. In another such patient (2), signs of insufficiency developed several days after omission of therapy in the second week of gestation, at a time when urinary 17-hydroxycorticoid excretion was normal. In a similar study in which therapy was withdrawn over a two-week period, cortisone and hydrocortisone were absent from the urine, but no untoward symptoms developed (39). Chromatographic studies of plasma (6) showed absence of cortisol in an untreated Addisonian patient in the third trimester of pregnancy. An increase in excretion of urinary neutral 17-ketosteroids above the nonpregnant level has been noted in several pregnant women with Addison's disease (1, 10, 23). Lack of urinary aldosterone has been demonstrated in

2 pregnant women previously subjected to bilateral adrenalectomy for Cushing's syndrome (13), and in 1 pregnant Addisonian patient (6). However, in another study of 2 bilaterally adrenalectomized pregnant women (14), an increase in urinary aldosterone during pregnancy was observed in one, whereas no rise was observed in the other. From these data it would appear that the placenta is not the source of the increased urinary aldosterone observed during normal pregnancy, yet it is conceivable that the placenta may furnish some of the material measured as urinary neutral 17-ketosteroids.

The findings on the patient reported here are consistent with the foregoing observations. It is possible that there was placental production of 17-ketosteroids during pregnancy, as manifested by maintenance of the levels of the total urinary 17-ketosteroids, androsterone and etiocholanolone during withdrawal periods throughout pregnancy, as contrasted with the drop in these levels during postpartum withdrawal periods. However, it would seem more likely that adrenal rests were present in this patient and that they were stimulated during pregnancy. This would be particularly suggested from the studies carried out eighteen months post partum. The latter data also afford an explanation for the levels of urinary and plasma 17-hydroxycorticoids during pregnancy following withdrawal of replacement therapy. It is possible that during pregnancy the degree of stimulation was greater than that induced by ACTH in the nonpregnant state. In addition, due regard must be paid to the delayed clearance of plasma corticoids during pregnancy. The marked fall in the urinary excretion of 17-hydroxycorticoids during the postpartum withdrawal period is probably a reflection of an incipient adrenal crisis provoked by the respiratory infection. No explanation can be offered for the normal plasma corticoid levels noted at this time. The small amount of aldosterone secreted both during and after pregnancy is probably in keeping with the patient's low level of residual adrenal function, and negates the likelihood of placental elaboration of aldosterone.

REFERENCES

1. JAILER, J. W., and KNOWLTON, A. I.: Simulated adreno-cortical activity during pregnancy in an Addisonian patient, *J. Clin. Invest.* **29**: 1430, 1950.
2. KAISER, I. H.: Electrolyte and steroid metabolism in a pregnant Addisonian, *J. Clin. Endocrinol. & Metab.* **16**: 1251, 1956.
3. RICHARDS, A.: Addison's disease and pregnancy, *Brit. M. J.* **1**: 421, 1952.
4. ROLLAND, C. F.; MATTHEWS, J. P., and MATTHEWS, G. D.: Pregnancy in Addison's disease, *J. Obst. & Gynaec. Brit. Emp.* **60**: 57, 1953.
5. WELLS, C. M., and WEST, J. E.: Management of Addison's disease during pregnancy, *Am. J. Obst. & Gynec.* **63**: 925, 1952.
6. BAULIEU, E. E.; DE VIGAN, M.; BRICAIRE, H., and JAYLE, M. F.: Lack of plasma

cortisol and urinary aldosterone in a pregnant woman with Addison's disease, *J. Clin. Endocrinol. & Metab.* **17:** 1478, 1957.

7. Moore, F. H., and Friedman, J. P.: Addison's disease and pregnancy, *Am. J. Obst. & Gynec.* **72:** 1340, 1956.
8. Singh, L. M.: Pregnancy in Addison's disease, *Brit. M. J.* **1:** 503, 1957.
9. Francis, H. H., and Forster, M. B.: Pregnancy in Addison's disease, *Proc. Roy. Soc. Med.*, **51:** 513, 1958.
10. Hunt, A. B., and McConahey, W.: Pregnancy associated with diseases of the adrenal glands, *Am. J. Obst. & Gynec.* **66:** 970, 1953.
11. Baulieu, E. E.; Bricaire, H., and Jayle, M. F.: Lack of secretion of 17-hydroxycorticosteroids in a pregnant woman with Addison's disease, *J. Clin. Endocrinol. & Metab.* **16:** 690, 1956.
12. Worner, K. G., and Mathew, A. G.: A case of pregnancy following bilateral adrenalectomy for Cushing's syndrome, *J. Obst. & Gynaec. Brit. Emp.* **63:** 248, 1956.
13. Laidlaw, J. C.; Cohen, M., and Gornall, A. G.: Studies on the origin of aldosterone during human pregnancy, *J. Clin. Endocrinol. & Metab.* **18:** 222, 1958.
14. Venning, E. H., and Beck, J.: Endocrine metabolic changes in the human during pregnancy, *in* Recent Progress in the Endocrinology of Reproduction, ed. by C. W. Lloyd. New York, Academic Press Inc., 1959, p. 345.
15. Holtorff, A. F., and Koch, F. C.: The colorimetric estimation of 17-ketosteroids and their application to urine extracts, *J. Biol. Chem.* **135:** 377, 1940.
16. Corcoran, A. C., and Page, I. H.: Methods for chemical determination of corticosteroids, *J. Lab. & Clin. Med.* **33:** 1926, 1948.
17. Silber, R. H., and Porter, C. C.: The determination of 17,21-dihydroxy-20-ketosteroids in urine and plasma, *J. Biol. Chem.* **210:** 923, 1954.
18. Ulick, S.; Laragh, J. M., and Lieberman, S.: Urinary metabolites of aldosterone, *Tr. A. Am. Physicians* **71:** 225, 1958.
19. Migeon, C. J.; Bertrand, J.; Wall, P. E.; Stempfel, R. S., and Prystowsky, H.: Metabolism and placental transmisison of cortisol during pregnancy near term, *Ciba Colloq. Endocrinol.* **11:** 338, 1957.
20. Rogoff, J. M., and Stewart, G. N.: Studies on adrenal insufficiency, *Am. J. Physiol.* **86:** 20, 1928.
21. Hills, A. G.; Venning, E. H.; Dohan, F. C.; Webster, G. D., and Richardson, E. M.: Pregnancy and adrenocortical function, *J. Clin. Invest.* **33:** 1466, 1951.
22. Gemzell, C. A.: Blood levels of 17-hydroxycorticosteroids in normal pregnancy, *J. Clin. Endocrinol. & Metab.* **13:** 898, 1953.
23. Bayliss, R. I. S.; Browne, J. C.; Round, B. P., and Steinbeck, A. W.: Plasma-17-hydroxycorticosteroids in pregnancy, *Lancet* **1:** 62, 1955.
24. Assali, N.; Garst, J. B., and Voskian, D.: Blood levels of 17-hydroxycorticosteroids in normal and toxemic pregnancies, *J. Lab. & Clin. Med.* **46:** 385, 1955.
25. Martin, J. D., and Mills, I. H.: The effect of pregnancy on adrenal steroid metabolites, *Clin. Sc.* **17:** 137, 1958.
26. Christy, N. P.; Gordon, W. E. L.; Longson, D.; Wallace, E. Z., and Jailer, J. W.: Some observations upon adrenal cortical function during pregnancy. Abstract No. 4, Program of Fortieth Meeting of The Endocrine Society, San Francisco, June 19–21, 1958 (p. 14).
27. Davis, R.: L'élimination des corticoïdes au cours de la grossesse, *Gynéc. et Obst.* (Paris) **53:** 57, 1954.
28. Wallace, E. Z.; Silverberg, H. I., and Carter, A. C.: Effect of ethinyl estradiol on

plasma 17-hydroxycorticosteroids, *Proc. Soc. Exper. Biol. & Med.* **95:** 805, 1957.
29. MIGEON, C. J.; BERTRAND, J., and WALL, P. E.: Physiological disposition of 4-C^{14}-cortisol during pregnancy, *J. Clin. Invest.* **36:** 1350, 1957.
30. DE COURCY, C.; GRAY, C. H., and LUNNON, J. D.: Adrenal hormones in human placenta, *Nature*, **170:** 494, 1952.
31. BERLINER, D. L.; JONES, J. E., and SALHANICK, H. A.: The isolation of adrenal-like steroids from the human placenta, *J. Biol. Chem.* **223:** 1043, 1956.
32. SALHANICK, H.; JONES, J. E., and BERLINER, D.: Sources of placental steroids, *Fed. Proc.* **15:** 160, 1956.
33. LITTLE, B., and ROSSI, E.: Production of Porter-Silber reactive material by human placenta incubated *in vitro*, *Endocrinology* **61:** 108, 1957.
34. TROSEN, P.: Production of free and conjugated corticosteroids by perfused human placenta, (abstract) *J. Clin. Invest.* **37:** 936, 1958.
35. MARTIN, J. D., and MILLS, I. H.: Aldosterone excretion in normal and toxemic pregnancies, *Brit. M. J.* **2:** 571, 1956.
36. VENNING, E. H.; PRIMROSE, T.; CALIGARIS, L. C. S., and DYRENFURTH, I.: Aldosterone excretion in pregnancy, *J. Clin. Endocrinol. & Metab.* **17:** 473, 1957.
37. GARDNER, L.: Quoted by Migeon C., in Discussion, *Ciba Colloq. Endocrinol.* **11:** 260, 1957.
38. HUIS IN'T VELD, L. G.: L'excrétion des 17-cétostéroïdes au cours de la grossesse, *Gynéc. et obst.* **53:** 42, 1954.
39. GRAY, C.: Discussion (Ref. 25).

METHOD FOR DETERMINATION OF URINARY 5β-PREGNANE-3α,17α,20α-TRIOL AND Δ⁵-PREGNENE-3β,17α,20α-TRIOL*

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ABSTRACT

During studies of adrenal function in human hypertension, Bongiovanni and Eberlein's procedure for determination of urinary pregnanetriol was found inaccurate. Pregnanetriol eluted from an alumina column with benzene-6% ethanol, after enzymatic and acid hydrolysis of the steroids in urine, was contaminated in most instances by 2 substances interfering with the determination in concentrated sulfuric acid. One of them was a compound described in a previous communication and identified as Compound III. The second was characterized as Δ⁵-pregnene-3β,17α,20α-triol. Modifications of the original procedure are proposed for the specific determination of 5β-pregnane-3α,17α,20α-triol and Δ⁵-pregnene-3β,17α,20α-triol. The modified method involves successive chromatographic separation of the crude neutral-extract residue of urine, first on a silica-gel column, second on an ethylene-glycol/toluene paper system, and finally on an alumina column. Spectrophotometric determination of the highly purified compound, free from interfering substances, is achieved by use of the sulfuric-acid color reaction.

IN ORDER to provide more information on the possible role of the adrenal cortex in human hypertension, the urinary excretion of various corticosteroids and their chief metabolites was investigated in normal subjects and in hypertensive patients (1–3). Using Bongiovanni and Eberlein's procedure (4), the mean urinary excretion of 5β-pregnane-3α,17α,20α-triol showed a statistically significant ($P < 0.01$) decrease in groups of patients with essential, renal and malignant hypertension as compared to the excretion in normal subjects (3).

The specificity and accuracy of this procedure were found inadequate in most instances because of the presence of at least one or two interfering substances. These were Compound III (previously described (5)) con-

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jugated as a glucuronide, and Δ^5-pregnene-3β,17α,20α-triol excreted most likely as a sulfate. Modifications of Bongiovanni and Eberlein's procedure are proposed for accurate specific determination of pregnanetriol and of Δ^5-pregnenetriol, based on isolation of these compounds in a high degree of purity.

MATERIALS

The reagents used were as follows:

1. Chloroform (Mallinckrodt, analytical reagent), redistilled and preserved with ethanol 0.1 per cent.
2. Ethanol (Gooderham and Worts), twice distilled.
3. Benzene (Mallinckrodt, analytical reagent), redistilled.
4. Acetone (Mallinckrodt, analytical reagent), redistilled.
5. Ethylene glycol (Fisher, reagent grade).
6. Toluene (Merck, reagent grade).
7. Methanol (Merck, reagent grade), redistilled.
8. Concentrated sulfuric acid (Merck, reagent grade).
9. Pyridine (Merck, reagent grade).
10. Acetic acid, glacial (Baker's analyzed).
11. Trichloracetic acid (Fisher, reagent grade).
12. Periodic acid, C. P. (Smith Chemical Co.).
13. Whatman #2 paper "especially selected for chromatography," and washed prior to chromatography in a Soxhlet apparatus for seventy-two hours with a 1:1 v/v mixture of benzene and methanol.
14. Glass wool, washed twice with benzene prior to use.
15. Silica-gel (Davison, grade 923, mesh 100–200), used without reactivation.
16. Activated alumina (Aluminum Company of America, grade F-20), used without reactivation.
17. Methone (5,5-dimethyl-1,3-cyclohexanedione), Eastman.

STUDY OF BONGIOVANNI AND EBERLEIN'S PROCEDURE

This procedure (4) for measurement of urinary 5β-pregnane-3α,17α,20α-triol involves beef liver β-glucuronidase hydrolysis, extraction of the hydrolyzed steroids with dichloromethane, and alumina-column chromatography of the crude neutral-extract residue. The prognanetriol fraction obtained in the benzene-6% methanol eluate is almost always contaminated by one or two other substances (see subsequent sections of this report). The lack of specificity of the concentrated sulfuric-acid color reaction leads to inaccurate results. One of these compounds has been previously described (5) and is referred to as Compound III. The other was found to be identical with Δ^5-pregnene-3β,17α,20α-triol, isolated by the Hirschmanns (6) and by Okada *et al.* (7) from the urine of adrenal carcinoma patients, and by Fotherby (8) from the urine of normal men.

Identity of Compound III with the previously isolated material was established by comparing all its known properties, excluding infrared spectrophotometry. This substance is present in most urines obtained

from normal subjects and hypertensive patients in amounts varying from a few micrograms to several milligrams per day, and its excretion is greatly influenced by the dietary potassium intake. The second interfering substance eluted in the same benzene-6% methanol fraction from the alumina column was present in all urinary aliquots studied. It reacts with tricholoroacetic-acid reagent (9) by producing an orange fluorescence when illuminated with an ultraviolet light source emitting at 366 mμ. Its spectrum in concentrated sulfuric acid closely resembles that of Δ^5-pregnene-3β,17α,20β-triol (Fig. 1). However, the latter compound is less polar in

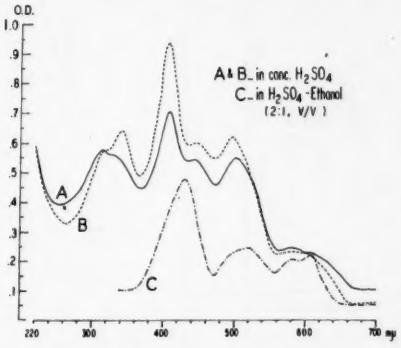

FIG. 1. Absorption spectra in concentrated sulfuric acid after two hours at room temperature, of Δ^5-pregnene 3β,17α,20α-triol (A) and Δ^5-pregnene 3β,17α,20β-triol (B). (C) is the spectrum of A in ethanol-sulfuric acid 1:2 v/v after fifteen minutes. (Beckman DK_2 recording spectrophotometer.)

the ethylene-glycol/toluene paper chromatographic system than is the urinary material (Fig. 2), although it reacts with trichloroacetic-acid reagent by producing the same orange fluorescence. The urinary material when oxidized with periodic acid yields acetaldehyde and dehydroisoandrosterone. The latter was identified by its mobility in Kochakian's propylene-glycol/benzene-cyclohexane system (10) as well as by a mixed melting point at 152–3°C., with the reference standard.[1] Acetaldehyde was identified by formation of a condensation product with methone (dimethyl-cyclohexanediol) (11). This derivative was crystallized in aqueous ethanol, and the mixed melting point taken with a sample obtained with freshly prepared acetaldehyde was 138°C. (hot stage). Assuming an uptake of 1 mole in the periodic-acid oxidation, this identifies positively a 17,20-dihydroxy,21-methyl side-chain. Formation of dehydro-isoandrosterone and acetaldehyde provides evidence for presence of a 3β-hydroxy-Δ^5 steroid skeleton with a 17,20 dihydroxy 21-methyl side-chain.

As this urinary material is more polar in the paper chromatographic systems used than is Δ^5-pregnene-3β,17α,20β-triol, it was postulated by analogy with the mobility of both α- and β-isomers of 5β-pregnane, 3α,17α,

[1] Kindly supplied through the courtesy of the CIBA Pharmaceutical Products, Inc.

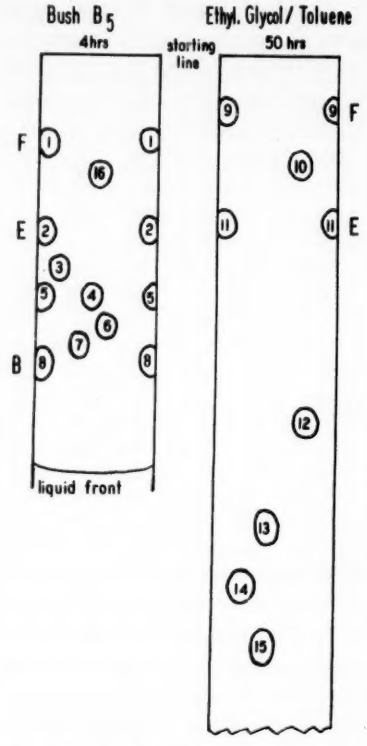

FIG. 2. Paper chromatographic mobilities:

1. Hydrocortisone in Bush B5 system, four hours at room temperature.
2. Cortisone in Bush B5 system, four hours at room temperature.
3. Δ^5-Pregnene-3β,17α,20α-triol in Bush B5 system, four hours at room temperature.
4. 5β-Pregnane-3α,17α,20α-triol in Bush B5 system, four hours at room temperature.
5. Caffeine in Bush B5 system, four hours at room temperature.
6. Δ^5-Pregnene-3β,17α,20β-triol in Bush B5 system, four hours at room temperature.
7. 5β-Pregnane-3α,17α,20β-triol in Bush B5 system, four hours at room temperature.
8. Corticosterone in Bush B5 system, four hours at room temperature.
9. Hydrocortisone in ethylene-glycol/toluene system, fifty hours at room temperature.
10. Compound III in ethylene-glycol/toluene system, fifty hours at room temperature.
11. Cortisone in ethylene-glycol/toluene system, fifty hours at room temperature.
12. Δ^5-Pregnene-3β,17α,20α-triol in ethylene-glycol/toluene system, fifty hours at room temperature.
13. 5β-Pregnane-3α,17α,20α-triol in ethylene-glycol/toluene system, fifty hours at room temperature.
14. Δ^5-Pregnene-3β,17α,20β-triol in ethylene-glycol/toluene system, fifty hours at room temperature.
15. 5β-Pregnane-3α,17α,20β-triol in ethylene-glycol/toluene system, fifty hours at room temperature.
16. Compound III in Bush B5 system, four hours at room temperature.

20α-triol, that this material could be identical with the Δ^5-pregnene-3β, 17α,20α-triol previously described by the Hirschmanns (6). The 20α-hydroxy isomer was expected to be more polar than the 20β-hydroxy isomer (Fig. 2). The urinary material after being crystallized three times out of 80 per cent methanol, melted at 226–7° C. The melting point previously reported with Δ^5-pregnene-3β,17α,20α-triol by the Hirschmanns (6) is 224–8° C., and by Butenandt, 227° C. (12).

After completion of the foregoing studies, we were fortunate to obtain a sample of authentic Δ^5-pregnene-3β,17α,20α-triol.[2] The mobility of our urinary material was identical to that of authentic Δ^5-pregnene-3β,17α, 20α-triol in both the paper chromatographic system of ethylene-glycol/ toluene and that of benzene/55% aqueous methanol (Bush B5). The mixed melting point of our material with the reference compound was 226–7° C.

When acetylated with acetic anhydride in pyridine, the urinary material yielded a product which, after crystallization in 80 per cent methanol, melted at 206–8° C. The Hirschmanns reported a melting point of 204–6° C. for the diacetylated derivative.

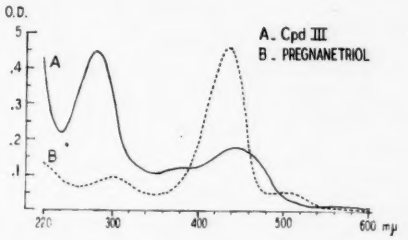

FIG. 3. Absorption spectra of Compound III (A) and 5β-pregnane-3α,17α, 20α-triol (B) in concentrated sulfuric acid after two hours at room temperature.

The results of infrared spectrophotometry of the prepared diacetate were identical to those for standard Δ^5-pregnene-3β,17α,20α-triol diacetate.[3]

Δ^5-Pregnene-3β,17α,20α-triol has a mobility very slightly lower than that of pregnanetriol in the Bush B5 system, but it is sufficiently more polar in the ethylene-glycol/toluene system to be well separated from pregnanetriol (Fig. 2).

The chromogenic spectrum of Compound III (5) in concentrated sulfuric acid shows a secondary peak at 450 mμ (Fig. 3), and that of Δ^5-pregnenetriol shows a maximum absorption at 406 mμ (Fig.1). They both interfere with the determination of pregnanetriol in concentrated sulfuric acid, as proposed by Bongiovanni and Eberlein. This interference is especially accentuated when the Allen correction is applied for calculation of pregnanetriol. Results may be too high or too low, dependent upon the proportion of the 3 substances present (Table 3). This is due to broadening of the pregnanetriol peak in sulfuric acid on one or both sides, or to the displacement of the absorption maximum (Fig. 4).

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[2] Kindly supplied by Dr. H. Hirschmann, Cleveland.

[3] Performed through the courtesy of Dr. Thomas Gallagher, Sloan-Kettering Institute, New York, N. Y.

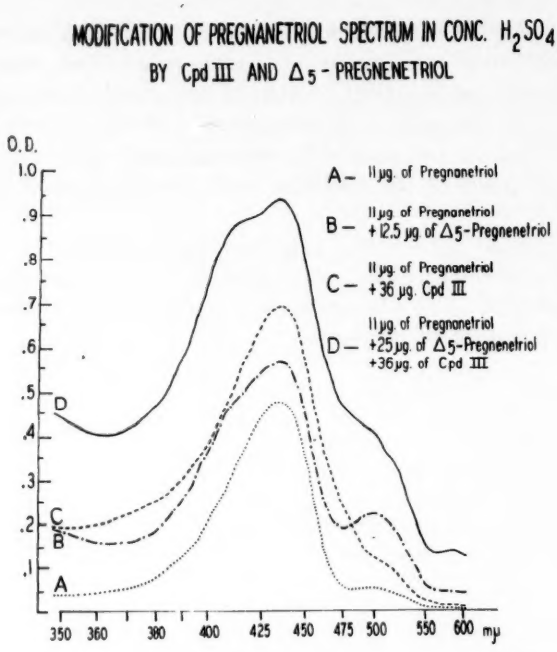

FIG. 4. Absorption spectra in 2 ml. of concentrated sulfuric acid after two hours at room temperature.

PROPOSED PROCEDURES FOR ISOLATION AND DETERMINATION OF 5β-PREGNANE-3α,17α,20α-TRIOL AND OF Δ⁵-PREGNENE-3β,17α,20α-TRIOL IN HUMAN URINE

5β-Pregnane-3α,17α,20α-triol

A 3-hour or 6-hour aliquot of a 24-hour collection of urine is incubated with beef liver β-glucuronidase (300 units per ml. of urine) at pH 4.8 and 37° C. for twenty-four hours, and subjected to extraction four successive times with 0.2 volume of chloroform. The pooled chloroformic extract is washed twice with 1 volume of 0.1 *N* sodium hydroxide and once with the same volume of water. Washings are back-extracted with an equal volume of chloroform. The chloroformic extracts are pooled and evaporated to dryness in a rotary type evaporator.[4] The residue of the crude neutral extract is chromatographed on a silica-gel column as previously described (13), using a 2.5-Gm. silica-gel column, 7 mm. in diameter. Elution is carried out with two successive mixtures, namely, chloroform:acetone 99:1 v/v (50 ml.) and chloroform:acetone 1:1 v/v (100 ml.). 5β-Pregnane-3α,17α,20α-triol is eluted quantitatively in the corticosteroid fraction by the latter eluate. The residue obtained from evaporation of this corticosteroid fraction is chromatographed on the ethylene-glycol/toluene system (14) for sixteen hours on a

[4] Flash-Evaporator, Laboratory Glass Supply, New York, N. Y.

strip of chromatographic paper 6 inches wide and 20 inches long, using cortisone as standard (Table 1).

The position of pregnanetriol is localized on the paper by cutting out a strip (2 mm. wide) from the center of the chromatogram and spraying it with the ethanolic trichloroacetic reagent[5] of De Courcy (9). After drying, the strip is heated for ten minutes in an oven at 75° C. Pregnanetriol reacts to produce a pale blue color in visible light and exhibits a greenish blue fluorescence under illumination with an ultraviolet light source emitting maximally at 366 mμ. Two micrograms can be detected by fluorescence and about 10 micrograms can be seen in visible light.

The pregnanetriol zone on the ethylene-glycol/toluene system is cut off and eluted with ethanol as previously described (15). The dry residue of this eluate is used for chromatographic separation on an alumina column prepared in a chromatographic tube

TABLE 1. MOBILITIES OF STEROIDS INVOLVED IN THIS INVESTIGATION IN THE BENZENE/55% AQUEOUS METHANOL SYSTEM OF BUSH (B5) AND THE ETHYLENE-GLYCOL/TOLUENE SYSTEM

Compound (numbered according to Fig. 2)	Bush B5, R_f	Ethylene-glycol/toluene (cortisone reference)
1, 9) Hydrocortisone	0.21	0.33
2) Cortisone	0.43	1.0
3, 12) Δ^5-Pregnene-3β,17α,20α-triol	0.51	2.19
4, 13) 5β-Pregnane-3α,17α,20α-triol	0.58	2.80
6, 14) Δ^5-Pregnene 3β, 17α,20β-triol	0.65	3.15
7, 15) 5β-Pregnane 3α,17α, 20β-triol	0.70	3.5
8) Corticosterone	0.74	—
10, 16) Compound III	0.34	0.56
5) Caffeine	0.58	—

7 mm. in diameter. A washed glass-wool plug serves as a support for the adsorbent. The tube is filled with benzene, and alumina is poured to the height of 7 cm. The benzene is left to percolate and the column is washed with 40 ml. of benzene. Since pregnanetriol is poorly soluble in benzene, the residue of the eluate from the paper chromatographic zone containing pregnanetriol is dissolved in 0.25 ml. of ethanol, to which is later added 50 ml. of benzene. This solution is transferred to the alumina column and the procedure is repeated. To assure complete dissolution of the residue, 1 ml. of ethanol is used to rinse the beaker which contains the residue, followed by the addition of 49 ml. of benzene. This fraction is transferred to the column and allowed to percolate. The use of the first two eluents in this manner permits the complete dissolution of the residue containing the pregnanetriol eluted from the paper. The third eluent, consisting of 100 ml. of benzene-6% methanol is then added to the column. The residue from the evaporation of this third eluent fraction from the alumina column is dissolved in 10 ml. of ethanol.

A 1-ml. aliquot of the dissolved residue is evaporated to dryness and a 2-ml. volume of concentrated sulfuric acid is added. The solution is left in the dark for two hours at room temperature. Absorbancy is determined at 435 millimicrons and the corresponding amount of pregnanetriol is read on a curve previously established with a standard compound.[6] The color produced over a concentration range from 5.5 μg. to 55.0 μg. in 2 ml.

[5] Ten grams of trichloroacetic acid dissolved in 20 ml. of ethanol.

[6] Kindly supplied by Dr. A. Bongiovanni, Philadelphia, Pa., and Dr. N. Brink, Merck Research Institute, Rahway, N. J.

of sulfuric acid follows Beer's law. Amounts of 5.5, 11.0 and 22.0 μg. show an optical density of 0.220, 0.458 and 0.899 respectively, using a Beckman spectrophotometer (Model DU) and 2-ml. silica cuvettes with a 10-mm. light path. In the presence of small quantities of pregnanetriol, aliquots larger than 1 ml. of ethanolic solution of the residue from the alumina column may be used, but when large quantities of pregnanetriol are present, a suitable dilution of the ethanolic solution must be made. The total quantity of the pregnanetriol is calculated according to the volume of the urine aliquot used and the dilution of the ethanolic solution of the residue from the alumina column fraction.

The identity of 5β-pregnane-3α,17α,20α-triol obtained from urine with the foregoing modified procedure was confirmed, after one crystallization in 80 per cent methanol, by a mixed melting point at 256–256.5° C. with authentic reference compounds (m.p., 256–256.5° C.)[7]

Recoveries of pregnanetriol added to three aliquots of the same urine were 88, 90 and 92 per cent of the added amount. This demonstrates excellent reproducibility of the procedure.

Δ⁵-Pregnene-3β,17α,20α-triol

The initial enzymatic hydrolysis and extraction procedures are the same as for pregnanetriol. The urine is then adjusted to pH 1 with concentrated hydrochloric acid and subjected to continuous extraction for forty-eight hours in a Cohen type extractor. Preliminary purification on a silica-gel column (Δ⁵-pregnenetriol is eluted in the corticosteroid fraction), paper chromatography in the ethylene-glycol/toluene paper system, and reaction with ethanolic trichloroacetic acid reagent are performed as for pregnanetriol. Δ⁵-Pregnenetriol is localized by the detection of an orange fluorescence after illumination of the paper with an ultraviolet light source emitting maximally at 366 mμ. In this system, Δ⁵-pregnenetriol is well separated from pregnanetriol. The zone containing Δ⁵-pregnenetriol is eluted with ethanol and its residue chromatographed on an alumina column in a way similar to that for pregnanetriol. Δ⁵-Pregnenetriol is also eluted in the benzene-6% ethanol fraction. To a suitable aliquot (usually $\frac{1}{10}$) of the dry residue a 2-ml. volume of concentrated sulfuric acid is added. The mixture is left in the dark for two hours at room temperature. Absorbancy is determined at 406 mμ and the corresponding amount of Δ⁵-pregnene-3β,17α,20α-triol is read on a curve established with Δ⁵-pregnene-3β,17α,20β-triol under identical conditions.[8] The optical densities determined with 10, 20 and 30 μg. of the steroid are 0.158, 0.314 and 0.471 respectively.

RESULTS

The results are listed in Table 2. Both normal subjects and hypertensive patients received unrestricted diets. Urine was collected from the normal females only during the first part of the menstrual cycle, to avoid the rise in pregnanetriol during the second phase of the cycle (16). This precaution was not taken for urine collected from the hypertensive women. As can be seen, the mean excretion in 19 normal subjects was 947 μg. per twenty-

[7] Kindly provided through the courtesy of Dr. W. Klyne, Medical Research Council, England.

[8] Kindly supplied by Dr. W. Klyne, Medical Research Council, England.

TABLE 2. URINARY EXCRETION OF 5β-PREGNANE-3α,17α,20α-TRIOL AND Δ^5-PREGNENE 3β,17α,20α-TRIOL IN HYPERTENSIVE AND IN NORMAL SUBJECTS

	Pregnanetriol		Δ^5-Pregnenetriol	
	No.	Mean excretion (μg./24 hrs.)	No.	Mean excretion (μg./24 hrs.)
Normal subjects	19	947 ± 116*	9	293 ± 56
Patients with essential hypertension	26	348 ± 72	5	99 ± 45
Patients with malignant hypertension	13	327 ± 90	4	173 ± 45
Patients with renal hypertension	8	134 ± 28	1	82

* Mean excretion and standard error of the mean.

four hours (S.E. ± 116). In all patients with hypertension, whether essential, renal or malignant, there was a markedly significant decrease in pregnanetriol excretion, especially in cases of malignant hypertension. In a 15-year-old girl with primary aldosteronism and malignant hypertension, the mean 24-hour excretion of pregnanetriol was 370 μg., and that of Δ^5-pregnenetriol was 277 μg. After total bilateral adrenalectomy, the level of both compounds (determined in two urine aliquots) was below 10 μg. per twenty-four hours. A comparison of the results obtained by Bongiovanni-Eberlein's method and by our modified procedure is shown in Table 3.

TABLE 3. COMPARISON OF URINARY PREGNANETRIOL VALUES (μG./24 HOURS) DETERMINED BY BONGIOVANNI-EBERLEIN'S METHOD AND OUR MODIFIED PROCEDURE

Normal subjects		Essential hypertension		Renal hypertension		Malignant hypertension	
B-E* method	Modified procedure	B-E* method	Modified procedure	B-E* method	Modified procedure	B-E* method	Modified procedure
1708	525	178	360	928	212	1408	306
1510	257	768	443	84	79	960	150
2919	1638	1305	503	45	23	328	81
		1464	572	3154	110	1072	445
		834	135	2919	62	960	653
		1098	1208	774	28	552	109
		1098	189			882	14
		864	39			804	124
		417	53			2196	67
		3612	1292				
		2334	1116				
		1510	185				
		1308	283				
		2448	328				
		252	144				
		534	128				
		768	9				
		876	116				
		192	467				
		2144	868				
		432	184				
		1988	303				

* Bongiovanni-Eberlein.

DISCUSSION

The specificity of this procedure is dependent upon the purity of the final product, or rather upon the absence of any other material than pregnanetriol (or of Δ^5-pregnenetriol (Fig. 2)) which produces a color reaction with concentrated sulfuric acid.

Introduction of the ethylene-glycol/toluene paper chromatographic system separates the only two substances which interfere in the sulfuric-acid reaction, namely, Δ^5-pregnene-3β,17α,20α-triol and Compound III. In this chromatographic system, the interference of Δ^5-pregnene-3β,17α,20β-triol, which is less polar than its 20α isomer, is also eliminated. As the pregnanetriol obtained from the alumina column is free from any interfering material, the Allen correction is not used and only one reading, at 435 mμ for pregnanetriol or at 406 mμ for Δ^5-pregnenetriol, is required for final determination.

When a small urinary aliquot is used, silica-gel column chromatography is not necessary and can therefore be omitted. The volume of the benzene-6% ethanol used for elution of the alumina column depends on the activity of the particular batch of alumina and should be adjusted according to the behavior of pure pregnanetriol or pure Δ^5-pregnenetriol on a similar column and using similar quantities. Thus it is not necessary to reactivate the aluminum oxide and to adjust the percentage of water.

After having been eluted from the paper chromatogram, pregnanetriol and Δ^5-pregnenetriol are difficult to dissolve in benzene. For this purpose, the ethanol which is to be used in the preparation of the first and second eluent for the alumina column should be employed. This is the reason why the residue is first dissolved in the ethanol portion of the eluting mixture to which is later added the benzene.

The chromogenic spectra of Δ^5-pregnene-3β,17α,20α-triol in concentrated sulfuric acid and in ethanol-sulfuric acid 1:2 v/v may be used for purposes of identification (17) (Fig. 1).

Compound III is more polar than cortisone in the ethylene-glycol/toluene system (Table 1) and can therefore be easily separated from pregnanetriol in the same system (Fig. 2). The mobility of pregnanetriol in the Bush B5 system is the same as that of caffeine, which can be used as a reference standard in this system.

During this study, aldosterone was also determined in the same urine aliquot as pregnanetriol. It should be emphasized that the urinary aliquots in the present study underwent both enzymatic and acid hydrolysis. As pregnanetriol is conjugated as glucuronide, acid hydrolysis is not mentioned in the procedure for this steroid. According to Fotherby (8), Δ^5-pregnene-3β,17α,20α-triol is likely conjugated as a sulfate; hence there is a

possibility that it may not interfere in the original procedure as described by Eberlein and Bongiovanni for the determination of pregnanetriol. To obtain sufficient separation of aldosterone, chromatography of the crude urinary neutral extract in the ethylene-glycol/toluene system was carried out for fifty hours on a chromatographic paper strip 6 inches wide and 40 inches long.

The method described is reasonably convenient for clinical use. Final estimation by the sulfuric-acid color reaction is preferred to procedures measuring the 17-ketosteroids or the aldehyde formed after oxidation of the glycol side-chain. These latter methods require the utmost care and are too difficult for routine use.

As far as we know, this is the first report of the quantitative measurement of Δ^5-pregnene-3β,17α,20α-triol in urine obtained from normal subjects and hypertensive patients.

REFERENCES

1. Genest, J.; Koiw, E.; Nowaczynski, W. J., and Leboeuf, G.: Further studies on urinary aldosterone in human arterial hypertension, *Proc. Soc. Exper. Biol. & Med.* **97:** 676, 1958.
2. Genest, J.; Nowaczynski, W. J.; Koiw, E., and Sandor, T.: Method for the isolation and determination of aldosterone and other steroids from urinary extracts. Fourth International Congress of Biochemistry, Vienna, September 1958. Supplement to International Abstracts of Biological Science, p. 116.
3. Genest, J.; Koiw, E.; Nowaczynski, W. J., and Sandor, T.: Study of a large spectrum of adrenocortical hormones in urines of normal subjects and hypertensive patients, *in* Proceedings of 32nd Session of American Heart Association, Philadelphia, October 1959, p. 700.
4. Bongiovanni, A. M., and Eberlein, W. R.: Critical analysis of methods for measurement of pregnane-3α,17α,20α-triol in human urine, *Analyt. Chem.* **30:** 388, 1958.
5. Nowaczynski, W. J.; Sandor, T.; Koiw, E.; Jones, R. N., and Genest, J.: Isolation of a steroid-like substance from urine and citrus fruits, *Canad. J. Biochem. & Physiol.* **36:** 869, 1958.
6. Hirschmann, H., and Hirschmann, F. B.: Steroid excretion in a case of adrenocortical carcinoma, *J. Biol. Chem.* **187:** 137, 1950.
7. Okada, M.; Fukushima, D. K., and Gallagher, T. F.: Isolation and characterization of 3β-hydroxy-Δ^5-steroids in adrenal carcinoma, *J. Biol. Chem.* **234:** 1688, 1959.
8. Fotherby, K.: The isolation of 3β-hydroxy-Δ^5-steroids from the urine of normal men, *Biochem. J.* **69:** 596, 1958.
9. De Courcy, C.: A trichloracetic acid reagent for the detection of pregnane-3α,17α,20-triols on paper chromatograms, *J. Endocrinol.* (London) **14:** 164, 1956.
10. Kochakian, C. D., and Stidworthy, G.: Paper chromatography of C_{19} steroids, *J. Biol. Chem.* **199:** 607, 1952.
11. Vorlander, D.: Methon als Aldehyd Reagens, *Ztschr. analyt. Chem.* **77:** 241, 1929.
12. Butenandt, A.; Schmidt-Thomé, J., and Paul H.: Umwandlung des Dehydro-

androsterons in 17-Iso-progesteron und Progesteron, *Ber. chem. Gesellsch.* **72:** 1112, 1939.

13. NEHER, R., and WETTSTEIN, A.: Physicochemical detection and measurement of aldosterone in body fluids and tissues, *Acta endocrinol.* **18:** 386, 1955.
14. NOWACZYNSKI, W. J., and KOIW, E.: A new paper chromatographic system for the separation of polar corticosteroids, *J. Lab. & Clin. Med.* **49:** 815, 1957.
15. NOWACZYNSKI, W. J.; KOIW, E., and GENEST, J.: Chemical method for the determination of urinary aldosterone, *Canad. J. Biochem. & Physiol.* **35:** 425, 1957.
16. PICKETT, M. T.; KYRIABIDES, E. G.; STERN, M. I., and SOMMERVILLE, I. F.: Urinary pregnanetriol throughout the menstrual cycle, *Lancet* **2:** 829, 1959.
17. GARSTENSTEN, H.; OERTEL, G. W., and EIK-NES, K. B.: Secretion of 17α-hydroxy-Δ^5-pregnenolone by the canine adrenal gland during stimulation with adrenocorticotropin, *J. Biol. Chem.* **234:** 2570, 1959.

JCEM

FAILURE TO ISOLATE CORTISOL FROM A PHEOCHROMOCYTOMA*

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ABSTRACT

A pheochromocytoma with an attached fragment of nontumorous adrenal tissue, removed from a 37-year-old woman, was studied by histochemical and incubation-chromatographic techniques. The normal adrenal tissue slices released, on the average, 48.4 μg. of hydrocortisone per Gm. (148.2 μg. after stimulation with ACTH); 17.0 μg. of cortisone (20.4 μg. after ACTH); and 5.4 μg. of aldosterone (2.8 μg. after ACTH). No steroid was released, however, by the slices of pheochromocytoma, even after stimulation with ACTH. A weak-to-moderate chromaffin reaction was observed throughout the tumor tissue.

THE isolation of cortisol from a tumor containing an excess of catecholamines and histologically resembling a pheochromocytoma, was recently reported by Mulrow, Cohn and Yesner (1). The tumor tissue, however, did not show the chromaffin reaction, and contained a few vacuolated cells with lipid-staining material in the cytoplasm. It appeared of interest to determine whether or not cells from a chromaffin-containing tumor could release steroidal substances *in vitro*.

In the present study, slices of a well differentiated pheochromocytoma from a clinically typical case were incubated, and the medium was analyzed for the presence of hydrocortisone, cortisone and aldosterone. The findings were compared with the data on hormonal release by nontumoral adrenal tissue excised from the same patient.

CASE REPORT

A 37-year-old, thin, white female was admitted to Hotel-Dieu Hospital of Montreal suffering from episodes of severe headaches with pallor, profuse sweating of the head and trunk, retrosternal tightness, palpitation, weakness and nervousness. These crises had increased in frequency and severity over a period of seven years.

While recumbent and in the resting state, her blood pressure fluctuated between

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140/90 and 180/110 mm. Hg, but rose as high as 250/130 during paroxysmal attacks, which were accompanied by severe tachycardia. There was no familial history of hypertension.

The results of Regitine and benzodioxane tests were repeatedly positive. The glucose tolerance curve was of the diabetic type. The excretion of total urinary catecholamines was elevated, the mean value being 1.63 mg. per twenty-four hours. Norepinephrine predominated, with a ratio to epinephrine of 27.6 to 1.0 (2). Excretion of urinary 17-hydroxycorticosteroids (3) was 2.3 mg. per twenty-four hours (normal range, 1.2–6.0 mg.).

Surgical exploration through an upper abdominal transversal incision demonstrated a right adrenal tumor. After excision of the mass with an adjacent portion of the right adrenal, the blood pressure and the output of urinary catecholamines declined to normal levels and have remained so after three months.

TUMOR AND METHODS OF STUDY

The well demarcated, ovoid, grey-pink tumor measured 2.9×2.5×2.0 cm. and, with a small fragment of attached adrenal tissue, weighed 18 Gm. It was sectioned into three portions. One part was fixed in Orth's fluid (9 volumes of 2.5 per cent potassium dichromate and 1 volume of 40 per cent formaldehyde solution) for subsequent pathologic examination; another part was frozen with dry ice and used for histochemical determinations; the central portion was used for incubation studies. A peripheral rim of tumor tissue, 3 mm. thick, was resected from this last portion, in order to avoid inclusions of cortical cells; furthermore, during slicing with the Stadie-Riggs microtome, 1 out of every 5 slices to be incubated was retained for histologic control.

Histologically, the tumor was composed of polyhedral cells disposed in clusters and small cords delineated by thin strands of connective tissue. The cells were large, with granular, slightly acidophilic cytoplasm and ill-defined borders. There were no vacuolated tumor cells, and fat stains (Sudan IV) yielded negative results. The nuclei were round and vesicular with occasionally prominent nucleoli. The tumor in some areas was lined by the capsule of the gland, but laterally was directly in contact with medullary and cortical tissue. A variable, weak-to-moderate, chromaffin reaction was observed throughout the mass. This reaction was, however, stronger and more uniform in the medullary tissue of the nontumoral gland.

The frozen portion of the tumoral tissue was placed in a cryostat and frozen sections were cut, 8μ thick. Triphosphopyridine nucleotide diaphorase (TPND) and glucose-6-phosphate dehydrogenase were determined by the technique of Scarpelli, Hess and Pearse (4, 5) using 2,2′-di-(p-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro BT) as the final electron acceptor. Intense activity of these two enzymes in the adrenal cortex of the rat has been demonstrated histochemically by Cohen (6). Recent histochemical studies in our laboratory on 8 surgical biopsy specimens of adrenal glands have confirmed this finding in human cortical tissue and have revealed in addition that, in the medulla, the enzymatic activity is weak. It was thought that this contrast in enzymatic pattern could help in the detection of inclusions of cortical cells in the pheochromocytoma.

Slices of the normal tissue (1.53 Gm.) and of the pheochromocytoma (3.58 Gm.) were incubated separately in Krebs-Ringer-bicarbonate-glucose medium (1 ml. per 100 mg. of tissue) in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide, at 37° C., in a Dubnoff metabolic shaking incubator. After a pre-incubation period of

thirty minutes, the slices of "normal" adrenal tissue were incubated for a period of two hours, and those of tumor tissue for a period of four hours. Both were then transferred to a fresh medium and re-incubated in the same manner in the presence of adrenocorticotropic hormone[1] (ACTH) in a concentration of 100 milliunits per 100 mg. of tissue.

The dry residue obtained after chloroform extraction of the medium, was passed through a silica-gel column (7). The corticosteroids eluted with the chloroform-acetone 1:1 fraction were separated in the Bush B5 paper chromatographic system (8). Hydrocortisone eluted directly from the Bush B5 system, and aldosterone and cortisone after re-chromatography in the Bush C system (8), were measured by their absorption of ultraviolet light at 240 mμ and by their reaction with isonicotinic acid hydrazide (9). For the tumor, a blue tetrazolium (BT) reaction was carried out on the whole paper strip, since no ultraviolet-absorbing spot was visible on the papergram under an ultraviolet light emitting maximally at 254 mμ.

TABLE 1. HORMONAL RELEASE BY NONTUMOROUS ADRENAL GLAND AND BY PHEOCHROMOCYTOMA (μG. PER GM. OF TISSUE INCUBATED PER HOUR)

Tissue	Hydrocortisone		Cortisone		Aldosterone	
		+ACTH		+ACTH		+ACTH
Nontumorous adrenal gland*	48.4	148.2	17.0	20.4	5.4	2.8
Pheochromocytoma	0.0	0.0	0.0	0.0	0.0	0.0

* Combined cortex and medulla.

RESULTS AND COMMENTS

A large amount of steroid was released by the normal (nontumorous) adrenal tissue (Table 1), the values being well above the normal range for this *in vitro* technique (10). After stimulation with ACTH, a threefold increase in cortisol production was observed.

In contrast, for the tumor tissue there was no evidence of ultraviolet-absorbing or α-ketolic reducing substances running on the chromatograms at the level of hydrocortisone, cortisone and corticosterone used marginally as reference standards. The BT reaction can detect less than 0.05 μg. of α-ketolic reducing steroid per square centimeter of paper surface (11). Since the purified extract from the incubation medium was deposited on a spot 5 mm. in diameter, any traces of hydrocortisone after five hours of running in the B5 system would have occupied, at most, 2 square centimeters of paper surface. This indicates that, if cortisol were produced by this tumor, it was less than 0.006 μg. per Gm. of tissue per hour, even after stimulation with ACTH.

The histochemical studies revealed weak activity of TPN diaphorase and glucose-6-phosphate dehydrogenase in the pheochromocytoma, con-

[1] Parke, Davis & Company, Lot No. 228781.

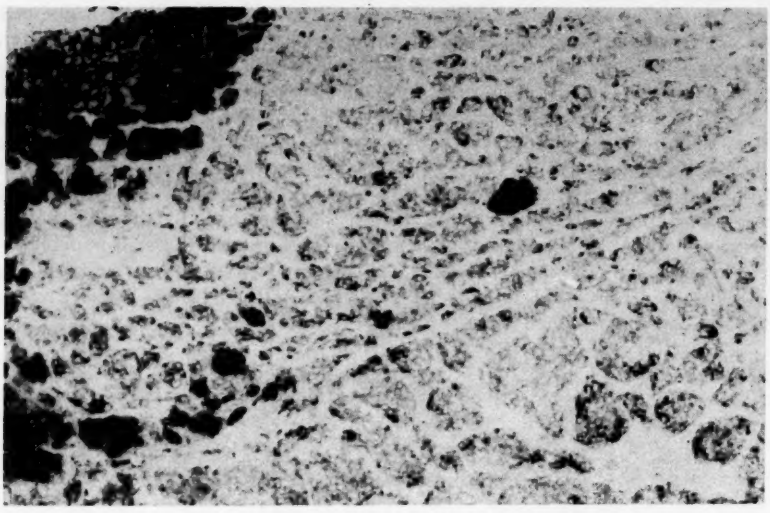

FIG. 1. TPN diaphorase activity. The pheochromocytoma is weakly reactive, but the adjacent cortex is strongly reactive. Islands of intensely stained cortical cells are included in the peripheral zone of the tumor. (×325.)

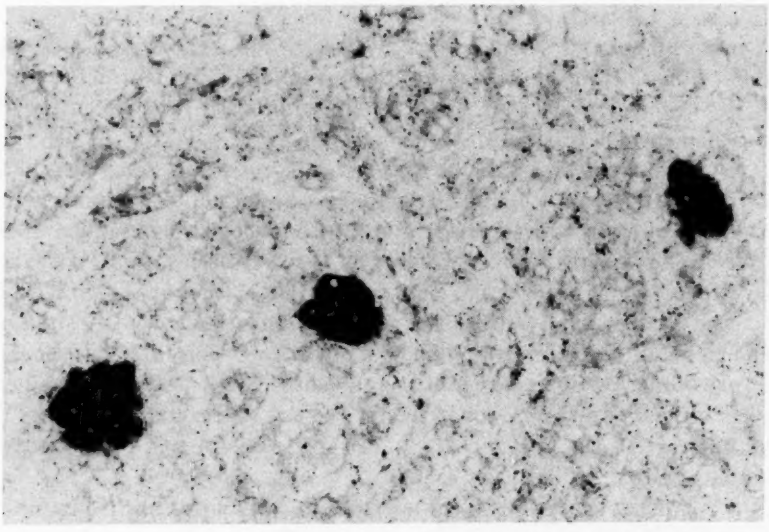

FIG. 2. Glucose-6-phosphate dehydrogenase activity. Three clusters of strongly reactive cortical cells are included in the weakly stained pheochromocytoma. The nuclei contain no enzyme and appear as clear round spaces. (×525.)

trasting with an intense activity in the adrenal cortex. Clusters of strongly reactive cortical cells were found embedded in the peripheral zone of the tumor. These cells could be detected in the histologic sections but were more readily identified in the histochemical preparations (Figs. 1 and 2). The cortical inclusions did not extend more than 1.3 mm. within the pheochromocytoma. They were undoubtedly not present in the incubated slices of tumor, because a peripheral rim of tissue measuring 3 mm. in thickness had been previously resected. Moreover, examination of the slices retained for histologic control failed to reveal any cortical cells.

The synthesis of cortisol observed in the tumor studied by Mulrow *et al.* may have been due to deep inclusions of cortical cells in the pheochromocytoma. Furthermore, in their tumor the results of the chromaffin test were negative. As emphasized by Karsner (12) (cited by Mulrow *et al.*), the histologic diagnosis of pheochromocytoma in the absence of a positive result with chromaffin stain is precarious. The present findings are at variance with those of Mulrow *et al.*, and suggest that a well differentiated, chromaffin-staining pheochromocytoma does not secrete corticosteroids.

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REFERENCES

1. Mulrow, P. J.; Cohn, G. L., and Yesner, R.: Isolation of cortisol from a pheochromocytoma, *Yale J. Biol. & Med.* **31:** 363, 1959.
2. Drujan, B. D.; Sourkes, T. L.; Layne, D. S., and Murphy, G. F.: The differential determination of catecholamines in urine, *Canad. J. Biochem. & Physiol.* **37:** 1153, 1959.
3. Peterson, R. E.; Karrer, A., and Guerra, S. L.: Evaluation of Silber-Porter procedure for determination of plasma hydrocortisone, *Analyt. Chem.* **29:** 144, 1957.
4. Scarpelli, D. G.; Hess, R., and Pearse, A. G. E.: The cytochemical localization of oxidative enzymes. I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase, *J. Biophys. & Biochem. Cytol.* **4:** 747, 1958.
5. Hess, R.; Scarpelli, D. G., and Pearse, A. G. E.: The cytochemical localization of oxidative enzymes. II. Pyridine nucleotide-linked dehydrogenases, *J. Biophys. & Biochem. Cytol.* **4:** 753, 1958.
6. Cohen, R. B.: Histochemical localization and metabolic significance of glucose-6-phosphate dehydrogenase system in adrenal cortex, *Proc. Soc. Exper. Biol. & Med.* **101:** 405, 1959.
7. Neher, R., and Wettstein, A.: Physicochemical detection and measurement of aldosterone in body fluids and tissues, *Acta endocrinol.* **18:** 386, 1955.
8. Bush, I. E.: Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues, *Biochem. J.* **50:** 370, 1952.

9. WEICHSELBAUM, T. E., and MARGRAF, H. W.: Determination of Δ^4-3-keto-corticosteroids in human peripheral plasma by the isonicotinic acid hydrazide (INH) reaction, *J. Clin. Endocrinol. & Metab.* **17**: 959, 1957.
10. DAVIGNON, J.: Studies on the "in vitro" release of corticosteroids by adrenal glands from normal subjects and hypertensive patients. Thesis for Master Degree of Science, McGill University, Montreal, August 1960.
11. NOWACZYNSKI, W.; GOLDNER, M., and GENEST, J.: Microdetermination of corticosteroids with tetrazolium derivatives, *J. Lab. & Clin. Med.* **45**: 818, 1955.
12. KARSNER, H. T.: Tumors of the adrenal. Atlas of Tumor Pathology, Section VIII, Fasc. 29. Washington, D. C., Armed Forces Institute of Pathology, 1950.

THYROIDITIS FOLLOWING ADMINISTRATION OF THYROID-STIMULATING HORMONE*

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ABSTRACT

The administration of 10 units of one lot of commercial thyroid-stimulating hormone (TSH) on two successive mornings to 21 healthy male adults produced acute thyroiditis in 10 of them. The thyroiditis was not accompanied or followed by the appearance of antibody reacting with the TSH used or with an extract of normal human thyroid, as measured by precipitin or hemagglutinin titers in serum. Hence these cases of acute thyroiditis did not appear to belong in the group of auto-immune reactions as seen in chronic thyroiditis. The occurrence of thyroiditis enhanced the net thyroidal uptake of I^{131} at forty-eight hours and raised the 24-hour and 48-hour conversion ratios (serum PBI^{131} as a percentage of the total serum radioactivity). Both groups of patients, *i.e.*, those with and without thyroiditis, manifested comparable increases in the serum PBI level. These increases, on the average, were of the order of magnitude observed in subjects given another lot of TSH which did not produce thyroiditis.

THE administration of one lot of thyroid-stimulating hormone (TSH) produced symptoms and signs of acute thyroiditis in approximately half of a group of 21 healthy euthyroid adults. This untoward reaction permitted evaluation of the effects of acute inflammation of the thyroid on the usual responses to exogenous TSH. The inflammatory reaction was not associated with increased hemagglutinin or precipitin titers to TSH or to an extract of normal human thyroid.

MATERIALS AND METHODS

The subjects were healthy males ranging in age from 20 to 50 years, all prison inmates. The net thyroidal uptake of I^{131}, the conversion to circulating PBI^{131} (expressed as the ratio of serum precipitable to total radioactivity of serum), and the level of serum protein-bound iodine (PBI) (1) were measured at intervals for forty-eight hours. One week later a dose of 10 units of lyophilized TSH (Armour Co., Lot #T 3412) freshly

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* This work was supported by grants from the Department of Health, Education and Welfare, and the John A. Hartford Foundation.

dissolved in 0.9 per cent sodium chloride solution was injected into the deltoid muscles (zero time) and again at twenty-four hours, when the measurements were repeated.

At each of the two testing sessions the subjects received approximately 50 microcuries of carrier-free I^{131} in capsule form. The emission gamma rays from the capsule and the radioactivity at 1 meter over the neck area, with and without a half-inch thick lead shield, were measured by means of a Nuclear Chicago scintillation detector using a sodium iodide thallium-activated crystal, a Baird atomic scaler, and a high voltage power supply. Readings were corrected for background values. The net thyroidal uptake was determined at five or six hours, twenty-four hours and forty-eight hours. Also, corrections were made for residual radioactivity over the thyroid and in the serum fractions prior to the repetitive studies with TSH.

After the thyroiditis had appeared, both pre-TSH and post-TSH sera were analyzed, by double diffusion in agar (2), for precipitating antibody reacting with the TSH used, and with an extract of normal human thyroid. The extract of thyroid was prepared by the method described by Witebsky *et al.* (3). Titers of hemagglutinating antibody to TSH and to the thyroid extract were also measured by the technique described by Boyden (4).

RESULTS

In the interval between six and thirty-six hours following the first injection of TSH, pain in the anterior neck with visible and palpable swelling of the thyroid developed in 10 of the 21 men. The degree of thyroiditis was graded as slight (1+), moderate (2+), or marked (3+). In the patients with 3+ thyroiditis the anterior neck area was hot, and fever and systemic prostration were present. The details of these findings are listed in Table 1. It should be emphasized that there was not necessarily any relationship between a reaction at the site of TSH injection and the presence of thyroiditis. Evidences of thyroiditis subsided in two to four days.

Comparisons of the net thyroidal uptake of I^{131}, the conversion ratio (serum PBI^{131} as the percentage of total serum radioactivity) and the serum PBI level in the subjects with and without thyroiditis are shown in Table 2 and Figure 1. The net uptake of I^{131} was increased at forty-eight hours in the patients with thyroiditis compared to those without thyroiditis. In the non-thyroiditis group, the TSH had little if any effect upon the uptake as established in these same patients one week earlier. The conversion ratios at twenty-four or forty-eight hours were not discernibly different in subjects with or without thyroiditis but they were greater than the ratios determined at a comparable time in other control subjects in this laboratory. The serum PBI levels rose to a comparable degree whether or not thyroiditis was present (Fig. 1). They were of the same magnitude as those observed in control studies with another lot (U 3810) of TSH.

Analyses of sera for precipitating antibodies and hemagglutinin titers with the lot of TSH used and with an extract of normal human thyroid showed no immediate nor late increase, either in the subject in whom thyroiditis had developed or those in whom it had not developed.

TABLE 1. CLINICAL FINDINGS IN HEALTHY MALES WITH AND WITHOUT THYROIDITIS FOLLOWING ADMINISTRATION OF TSH

Subject	Reaction at site of TSH injection	Clinical findings		Degree of thyroiditis
		Anterior neck	Thyroid	
W.B.	None	Tender, hot	Palpable	3+
R.M.	None	Tender, hot	Palpable	3+
J.C.	1+	Tender ±	Palpable	2+
H.S.	None	None	Palpable	1+
J.Cl.	Sore	Tender	Palpable	1+
G.G.	None	Tender	Palpable	1+
A.R.	None	Tender	Palpable	1+
V.S.	Sore	None	Palpable	1+
J.S.	None	Tender ±	Palpable	1+
S.C.	1+	None	Palpable	1+
M.V.	None	None	Not palpable	0
J.V.	None	None	Not palpable	0
J.Po.	1+	None	Not palpable	0
J.B.	None	None	Not palpable	0
J.P.	None	None	Not palpable	0
G.W.	Swollen	None	Not palpable	0
N.W.	Sore	None	Not palpable	0
G.C.	None	None	Not palpable	0
Z.P.	None	None	Not palpable	0
A.R.	None	None	Not palpable	0
J.K.	1+	None	Not palpable	0

DISCUSSION

The nature of the thyroiditis which occurred in 10 of 21 healthy adults receiving a particular lot of TSH remains unexplained. These men had been drawn at random, none gave a history of allergy, and none had received TSH previously. It may be that the initial reaction was of the so-called foreign-protein type and that it was most intensely manifest in the

TABLE 2. THYROIDAL UPTAKE OF I^{131} AND CONVERSION RATIOS IN SUBJECTS WITH AND WITHOUT THYROIDITIS FOLLOWING TREATMENT WITH TSH

Subjects	Net thyroidal uptake of I^{131} at 48 hours (% dose)	Conversion ratio (%)*	
		at 24 hours	at 48 hours
Without thyroiditis Control (1 wk. prior to TSH)	†24.79 ± 7.81 (9 cases)		
TSH (after 10 units at zero hour)		26.0 ± 14.5 (9 cases)	
TSH (after additional 10 units at 24 hours)	25.76 ± 10.68 (9 cases)		37.2 ± 16.6 (8 cases)
With thyroiditis Control (1 wk. prior to TSH)	25.28 ± 5.0 (10 cases)		
TSH (after 10 units at zero hour)		27.4 ± 19.0 (10 cases)	
TSH (after additional 10 units at 24 hours)	**35.93 ± 10.21 (10 cases)		37.4 ± 15.5 (10 cases)

* In a group of subjects (other than these listed) who had not received TSH, the conversion ratio was 8.1 ± 5.6 per cent (18 cases) and 29.7 ± 16.7 per cent (14 cases) at 24 and 48 hours, respectively.
** Difference between this value and the control is statistically significant.
† Mean and standard deviation.

thyroid because of the tropic properties of TSH. This reaction, in turn, could have released thyroglobulin into the circulation with a rapid production of auto-immune antibodies of the type observed in Hashimoto and related types of thyroiditis. This sequence is not supported, however, by the measurements of precipitins or hemagglutinins in the sera. The origin of the thyroiditis therefore remains obscure.

Inflammation in the gland was associated with a late increase in the net thyroidal uptake of radioactive iodine. This could have been the result of

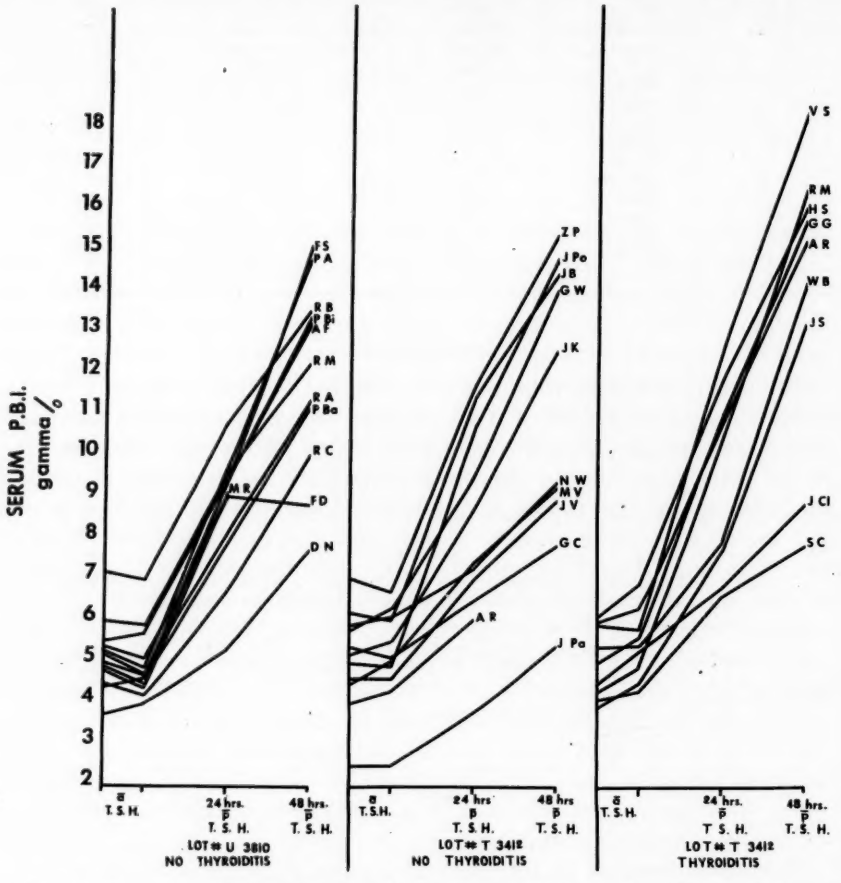

FIG. 1. Serum protein-bound iodine levels (μg. per 100 ml.) in subjects with and without clinical thyroiditis following administration of thyroid-stimulating hormone. Whether or not the TSH induced thyroiditis, the increases in the serum PBI level were comparable. The differences were not statistically significant.

increased vascularity of the gland, blockade of hormone synthesis, greater storage of formed hormone, or inhibition of its release. The conversion ratio and PBI values exclude the last of these possibilities and establish that hormone synthesis and release did occur.

The data suggest that this particular lot of TSH had either an earlier or a more pronounced effect, or both, on the thyroidal release of PBI^{131} to the circulation than it did on the I^{131} uptake. This deduction is based on the greater rise in the conversion ratio without any discernible effect on the thyroidal I^{131} uptake in subjects who exhibited no clinical thyroid reaction.

REFERENCES

1. Danowski, T. S.; Johnston, S. Y., and Greenman, J. H.: Alterations in serum iodine fractions induced by the administration of inorganic iodide in massive dosage, *J. Clin. Endocrinol. & Metab.* **10:** 519 (May) 1950.
2. Ouchterlony, Ö.: Antigen-antibody reactions in gels, *Arkiv Kemi. Mineral. Geol.* **B26:** 1–9, 1949. Cited in Methods in Medical Research, ed. by A. C. Corcoran. Chicago, Year Book Publishers, Inc., 1952, vol. 5, p. 377.
3. Witebsky, E.; Rose, N. R., and Shulman, S.: Studies on organ specificity. I. The serological specificity of thyroid extracts, *J. Immunol.* **75:** 269 (Oct.) 1955.
4. Boyden, S. V.: The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera, *J. Exper. Med.* **93:** 107 (Feb.) 1951.
5. Witebsky, E.; Rose, N. R.; Terplan, K.; Paine, J. R., and Egan, R. W.: Chronic thyroiditis and autoimmunization, *J.A.M.A.* **164:** 1439 (July 27) 1957.

Letters to the Editor

IODIDE GOITER

To The Editor:

The paper entitled "Iodide Goiter" by Paris *et al.* in the January 1960 issue of the Journal contains the following statement on page 64:

"In the differential diagnosis of an iodide goiter, Hashimoto's thyroiditis always should be considered. The case reported by Hydovitz and Rose illustrates this point.

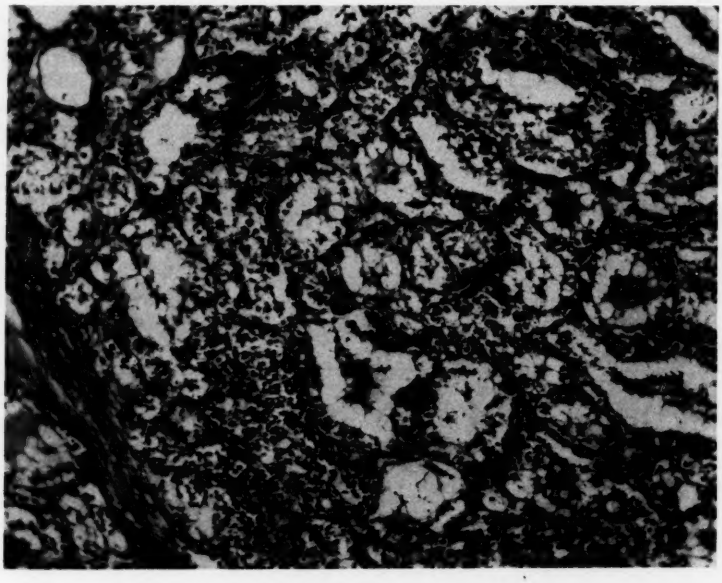

Figure 1

It concerns a 52-year-old woman under protracted iodide therapy for bronchial asthma whose goiter and myxedema were thought to be caused by the iodides. No biopsy material was available to substantiate the diagnosis. However, factors favoring the diagnosis of Hashimoto's thyroiditis with concomitant use of iodides included 1) a high thyroidal uptake of I^{131} and rapid decay of thyroidal radioactivity two months after the withdrawal of iodides, 2) normal and high normal values for serum PBI concentration attended by low amounts of BEI after a prolonged period of iodide withdrawal, and 3) the fact that myxedema disappeared only partially and the goiter not at all after the

withdrawal of iodides, with myxedema recurring when use of triiodothyronine was discontinued one year after the last dose of iodides."

The foregoing paragraph contains an inaccurate statement and an erroneous inference, which require correction. Our patient was a 52-year-old man. He died suddenly on September 4, 1956, shortly after the publication of our case report in the August 1956 issue of the Journal. At the time of his death he had been taking 180 mg. of desiccated thyroid daily for about eight months, following a previous protracted period of triiodothyronine therapy. Necropsy, performed by the Office of the Medical Examiner of Philadelphia, showed diffuse nodular enlargement of the thyroid. As will be seen in the accompanying photomicrograph (Fig. 1), there were scattered areas of hyperplasia and involution, but no evidence of Hashimoto's thyroiditis.

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March 28, 1960

A TEST OF PITUITARY RESERVE UTILIZING INTRAVENOUS SU-4885, WITH A NEW METHOD FOR EXTRACTION OF 11-DESOXYCORTICOSTEROIDS

TO THE EDITOR:

A much needed test of pituitary reserve has recently been developed by Liddle *et al.* (1). This test utilizes inhibition of 11β-hydroxylation (2) in the adrenal synthesis of cortisol by the administration of the compound, SU-4885, (2-methyl-1,2-bis(3-pyridyl)-1-propanone). Patients with an intact pituitary-adrenal axis respond to administration of SU-4885 with a drop in the concentration of plasma cortisol followed by an increase in the concentrations of plasma 11-desoxycortisol and desoxycorticosterone and an increase in the urinary excretion of tetrahydro-11-desoxycortisol and tetrahydrodesoxycorticosterone (2, 3). Since pituitary release of adrenocorticotropin (ACTH) is not inhibited by 11-desoxycorticosteroids (11-

DO-CS), the drop in plasma cortisol concentration results in an intense stimulus to the release of ACTH in normal subjects. However, the adrenal response is limited to the production of 11-DO-CS. In contrast, patients with inadequate pituitary function cannot effect an increase in ACTH production, and they do not respond with significant increases in plasma or urinary 11-DO-CS. The major 17-hydroxycorticosteroid excreted in normal subjects following administration of SU-4885 is tetrahydro-11-desoxycortisol (2, 3). However, significant amounts of normal adrenal excretory products (*e.g.*, tetrahydrocortisol and tetrahydrocortisone) are also present (4). Previous methods for evaluation of the adrenal response to SU-4885 have utilized the determination of 17-hydroxycorticosteroids or 17-ketogenic steroids. These methods, however, include the measurement of both the normal adrenal excretory products and the 11-DO-CS stimulated by SU-4885 and, therefore, reduce the sensitivity and specificity of the test.

Recent work in our laboratory has demonstrated that carbon tetrachloride has the unique property of extracting tetrahydro-11-desoxycortisol (tetrahydro-S) from aqueous media. Silber and Busch have shown that carbon tetrachloride does not extract tetrahydrocortisol or tetrahydrocortisone (5). Chromatographic analyses in our laboratory of carbon tetrachloride extracts of urine from patients treated with infusions of SU-4885 have demonstrated that tetrahydro-11-desoxycortisol is the only Porter-Silber chromogen extracted. These preliminary observations formed the basis of the method described here. The purpose of this communication is to present a new technic for the analysis of 11-DO-CS, and to present the findings in a group of normal subjects as well as in patients with various diseases of the pituitary-adrenal axis.

METHODS

All subjects received an infusion of 5 Gm. of SU-4885 as the ditartrate salt[1] in 500 ml. of isotonic sodium chloride. Aqueous heparin (10 mg.) was added to the infusion to prevent occasional mild local thrombophlebitis. The infusion was continued at a constant rate over a four-hour period from 8:00 a.m. to 12:00 noon. A 24-hour urine specimen was collected and an aliquot was frozen until the analysis could be performed. One milliliter of urine was then incubated with 100 units of bacterial glucuronidase (Sigma) suspended in 4 ml. of 1/15 *M* phosphate buffer at pH 6.5 for seventeen hours at 37° C. The hydrolyzed urinary steroids were extracted with 25 ml. of carbon tetrachloride, and the extract washed with 2 ml. of 0.1 *N* sodium hydroxide. The steroids were measured by the Porter-Silber color reaction. Tetrahydro-11-desoxycortisol[2] standards were added to the patient's control urine and treated in the same manner as the unknown sample.

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[1] Kindly supplied by Dr. C. H. Sullivan of CIBA Pharmaceutical Products Inc.

[2] Kindly supplied by Dr. John J. Schneider, Jefferson Medical College of Philadelphia.

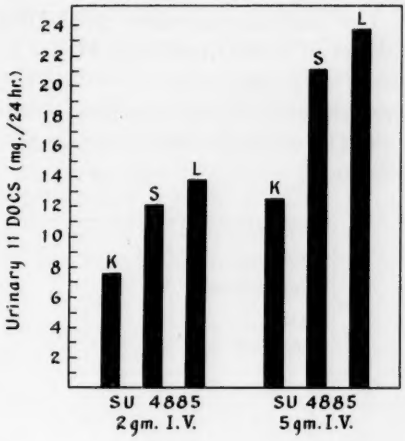

FIG. 1. Comparison of the urinary excretions of 11-DO-CS following four-hour infusions of 2 Gm. and 5 Gm. of SU-4885 in in each of 3 patients. At least three days elapsed between infusions. In Patient S the larger dose was given first.

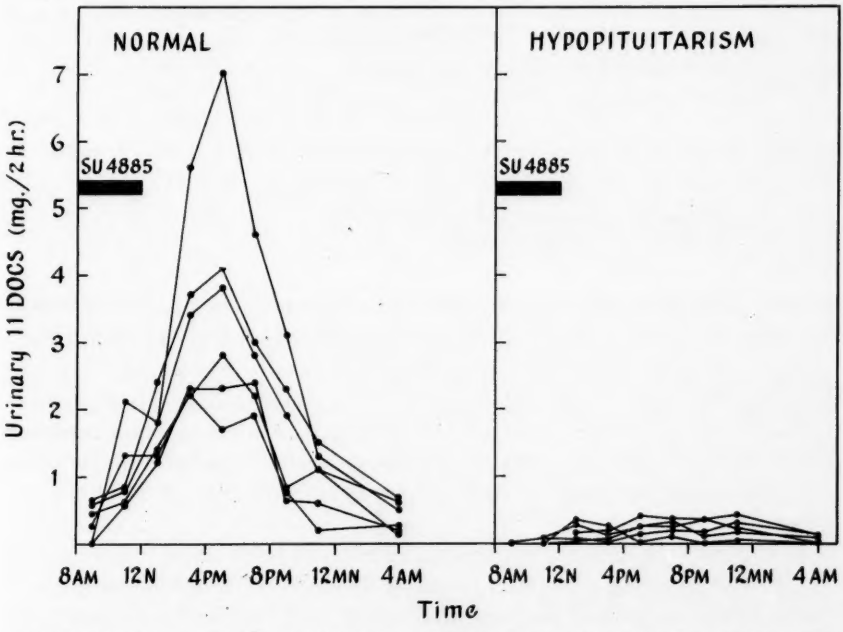

FIG. 2. Urinary excretion of 11-DO-CS at two-hour intervals following a 5-Gm. four-hour infusion of SU-4885 in 6 normal subjects and in 5 patients with untreated hypopituitarism.

RESULTS

The 24-hour excretion of 11-DO-CS was markedly influenced by the dose of SU-4885 ditartrate. Figure 1 shows the effect of a 5-Gm. dose compared with that of a 2-Gm. dose in 3 normal subjects. In each instance a greater response was obtained with the larger dose. The 2-Gm. dose resulted in a mean urinary excretion of 11.2 mg. per twenty-four hours as compared with 19.2 mg. for the 5-Gm. dose. Because of this consistent

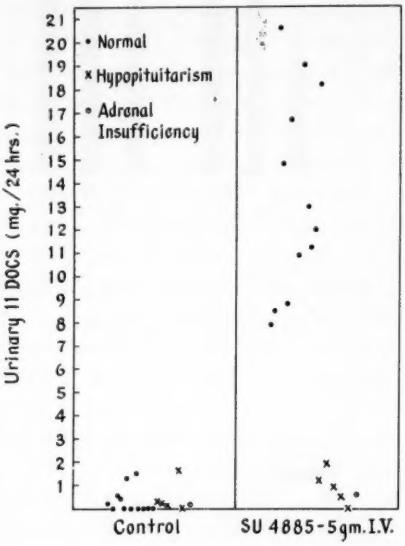

FIG. 3. Comparison of the 24-hour urinary excretions of 11-DO-CS on a control day and following a four-hour infusion of SU-4885 in 12 normal subjects, 5 patients with untreated hypopituitarism, and 1 patient with untreated adrenal insufficiency.

finding, all further tests were carried out with the 5-Gm. dose of SU-4885, an amount greater than that heretofore recommended in the literature.

Figure 2 shows the results in 6 normal subjects in whom the urine collections were made every two hours. The maximal urinary 11-DO-CS response occurred between 2:00 p.m. and 8:00 p.m. in all cases. This response was in marked contrast to that in 5 patients with untreated hypopituitarism (also shown in Figure 2) in whom no significant effect of SU-4885 was observed.

In Figure 3 the data are summarized on the 24-hour urinary excretion of 11-DO-CS following a 5-Gm. infusion of SU-4885 in 12 normal subjects, 5 patients with untreated panhypopituitarism, and 1 patient with untreated adrenal insufficiency. All subjects excreted less than 2 mg. of 11-DO-CS during the 24-hour control period. Following the infusion of SU-4885, all normal subjects excreted more than 8.0 mg. of 11-DO-CS per twenty-four

hours, whereas the patients with untreated pituitary and adrenal insufficiency excreted less than 2.0 mg. per twenty-four hours.

It is the purpose of this preliminary report to present data on a safe, reliable clinical test of the pituitary-adrenal axis, and to suggest a new extraction technic for the determination of 11-DO-CS which improves the differential diagnosis between normal subjects and patients with impaired pituitary reserve.

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REFERENCES

1. LIDDLE, G. W.; ESTEP, H. L.; KENDALL, J. W., JR.; WILLIAMS, W. C., JR., and TOWNES, A. W.: Clinical application of a new test of pituitary reserve, *J. Clin. Endocrinol. & Metab.* **19:** 875 (Aug.) 1959.
2. LIDDLE, G. W.; ISLAND, D.; LANCE, E. M., and HARRIS, A. P.: Alterations of adrenal steroid patterns in man resulting from treatment with a chemical inhibitor of 11β-hydroxylation, *J. Clin. Endocrinol. & Metab.* **18:** 906 (Aug.) 1958.
3. JENKINS, J. S.; POTHIER, L.; REDDY, W. J.; NELSON, D. H., and THORN, G. W.: Clinical experience with selective inhibition of adrenal function, *Brit. M. J.* **1:** 398 (Feb.) 1959.
4. GOLD, E. M.; DIRAIMONDO, V. C., and FORSHAM, P. H.: Quantitation of pituitary corticotropin reserve in man by use of an adrenocortical 11-beta hydroxylase inhibitor (SU-4885), *Metabolism* **9:** 3 (Jan.) 1960.
5. SILBER, R. H., and BUSCH, R. D.: The specificity of the reaction of phenylhydrazine with 17,21-dihydroxy-20-ketosteroids, *J. Clin. Endocrinol. & Metab.* **15:** 505 (Apr.) 1955.

The Endocrine Society

The 1961 Annual Meeting

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

The Scientific Sessions will be held from 9:00 a.m. to 5:00 p.m. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 23, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on, directly through the A. M. A. Housing Bureau. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1961.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Roy Hertz, National Cancer Institute, Bethesda, Maryland, not later than *February 15, 1961.* It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately 200 abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS; OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed 200 words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulae cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed 15 words;
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 inch filing cards, one for each author, giving his name and initials in the upper right-hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors, and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Hertz.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

The 1961 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

FRED CONRAD KOCH AWARD

In 1957 a substantial legacy was bequeathed to the Society by the late

Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

The American Goiter Association

The 1961 Annual Meeting

The next Annual Meeting of the American Goiter Association will be held in the Warwick Hotel, Philadelphia, Pennsylvania, *May 3–6, 1961.* Arrangements are being made for a Clinical Day in the Philadelphia Hospitals on May 3. Scientific papers will be presented in the Warwick Hotel on May 4, 5 and 6. For further information address the Secretary, John C. McClintock, M.D., 702 Madison Avenue, Albany 8, N.Y.

The Van Meter Prize Award for 1961

The American Goiter Association, Inc. again offers the Van Meter Prize Award of $300.00 to the essayist submitting the best manuscript of original and unpublished work concerning "Goiter—especially its basic cause." The studies so submitted may relate to any aspect of the thyroid gland in all of its functions in health and disease. The Award will be made at the Annual Meeting of the Association in the Warwick Hotel, Philadelphia, Pennsylvania, May 3–6, 1961. A place on the program will be reserved for the winning essayist if he can attend the meeting. When more than one author's name appears on the manuscript the authors will be asked to designate a single recipient to receive the award.

The competing essays may cover either clinical or research investigations, should not exceed 3,000 words in length and must be presented in English. Duplicate typewritten copies, double spaced, should be sent to the Secretary; John C. McClintock, M.D., 702 Madison Avenue, Albany 8, N. Y., *not later than January 1, 1961.* Manuscripts that do not conform to these requirements will not receive consideration. The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

Book Register

Cellular Aspects of Immunity. Proceedings of a CIBA Foundation Conference, Chantilly, France (Chairman, PROFESSOR R. KOURILSKY (Paris). Edited by G. E. W. WOLSTENHOLME, M.B., M.R.C.P., and M. O'CONNOR, B.A. Thirty-two participants from all over the world presented data on the dynamics of mesenchymal cells as modified by immunologic processes, including papers on the nomenclature of immunologically competent cells, theories of immunologic tolerance, the cellular basis of immunologic memory, delayed reactions and antibody formation as parallel types of cellular response, transfer of delayed hypersensitivity with leukocyte extracts in man, the earliest manifestations of specific immunity and hypersensitivity in neonatal mammals, multiple antibodies to cell constituents in systemic lupus erythematosis and related disorders, serum proteins related to immunity, and the *in vitro* action of antigen-antibody complexes on thrombocytes and erythrocytes; 495 pages; 117 illustrations; 1960. Little, Brown & Company, Boston, Mass. Price $10.50.

Die zellkernmorphologische Geschlechtserkennung in Theorie und Praxis. By DR. HERMANN ADOLF HIENZ, Privatdozent für allgemeine Pathologie und spezielle pathologische Anatomie, with a foreword by PROF. DR. E. RANDERATH, Direktor des Pathologischen Institutes der Universität Heidelberg. Volume 10 of "Einzeldarstellunger aus Theorie und Klinik der Medizin" edited by PROF. DR. HANS SCHAEFER, Heidelberg. (In German.) Special importance is given to the relationship between cell structure and function. Results obtained with the newer research methods (*e.g.*, phase-contrast microscopy and electron microscopy) are included, along with those obtained by standard histochemical methods. There is also a chapter on cells of the placenta, particularly in cases of abortion, and on cellular sex identification in living and dead newborn infants; a chapter on the cells of tumors, including teratoma and prostatic and mammary carcinoma; and a chapter on the sex chromatin patterns in various endocrine abnormalities such as Klinefelter's syndrome, Turner's syndrome, cryptorchism, hypophyseal dwarfism, eunuchoidism, male pseudohermaphrodism, feminizing testes, the adrenogenital syndrome, true hermaphrodism and agonadism and psychosexual conditions; paper; 219 pages; 40 illustrations; 27 tables; 1959. Dr. Alfred Hüthig Verlag, Heidelberg, Germany. Price, DM 30.

Elektrolyt-Kompendium. Physiologie, Pathologie und Therapie des Wasser- und Elektrolythaushalts. By DR. WERNER FLEISCHER, Department of Pathology, Veterans Administration Hospital, Milwaukee (Wood), Wisconsin and DR. ERNST FRÖHLICH, IV Medizinische Abteilung, Landeskrankenhaus, Graz. (In German.) Theoretical problems and practical applications are brought into relationship. The chapters of the book cover the following subjects: distribution of body water between cells and capillaries; osmotic dynamics; the functions of the electrolytes (anions and kations); homeostatic mechanisms including the role of the kidney; pathologic routes of water and electrolyte interchange; effects of changes in volume and concentration; acidosis and alkalosis; specific changes in sodium and potassium metabolism; clinical manifestations of disturbances in electrolyte balance; laboratory diagnostic data; basis of therapy; water and electrolyte balance in various endocrine diseases, including diabetic acidosis; cardiorenal problems and edematous conditions; and the importance

of water and electrolyte balance in surgery and pediatrics; 388 pages; 30 figures; 38 tables; 1960. Benno Schwabe & Co., Basel, Switzerland. In the U.S.A., Intercontinental Medical Book Corporation, 381 Fourth Ave., New York 16, N. Y. Price $9.50.

Klinische Physiologie. Aktuelle Probleme in Übersichten. By DR. W. A. MÜLLER, Stuttgart. Volume 1, Part 2 (in German). *Atypische Proteine* by VON H. CLEVE, H. DEICHER, F. HARTMANN and N. LANG covers nonpathogenic and pathogenic varieties of protein abnormalities in blood, with special reference to hemoglobin, plasma globulins, albumin and antigens. The changes associated with carcinomatous, fibroid and amyloid disease are discussed; 17 figures; 8 tables; 397 references. *Grundmechanismus der Magensäureproduktion und deren Regulation* by VON E. HEINZ deals with factors affecting gastric secretion, the mechanism of the secretion of acid, the transport of chloride and water, and the role of enzymes; 20 pages; 5 figures; 74 references. Published under the editorial auspices of *Deutschen medizinischen Wochenschrift*, 1960. Georg Thieme Verlag, Herdweg 63, Stuttgart, Germany. In the U.S.A., Intercontinental Medical Book Corporation, 381 Fourth Ave., New York 16, N. Y. Prices: broschiert DM 18; Vorzugspreis DM 15.30—U.S.A., $4.30 and $3.65.

Transactions of the Josiah Macy, Jr. Foundation Conferences. Publications Office, 16 West 46th Street, New York 36, N. Y.

Cold Injury. Sixth Conference, July 6–10, 1958, U. S. Army Medical Research Laboratory, Fort Knox, Ky. Edited by STEVEN M. HORVATH, M.S., PH.D., Division of Research, Lankenau Hospital, Philadelphia, Pa. Chairman, JOHN H. TALBOTT, M.D., Editor, Journal of the American Medical Association, Chicago, Illinois. The subjects covered are: Reducing a Large Efficient Homotherm to a Poikilothermic Status; Physiological Aspects of Hibernation in Mammals; Living on the South Polar Ice Cap; Medical Experiences at McMurdo Sound; Physiological Studies in the Antarctic; An Evaluation of Wind Chill; Shivering and Nonshivering Heat Production in Animals; The Problem of Equivalence of Specific Dynamic Action; Exercise Thermogenesis and Cold Thermogenesis; Energy Balance in Cold Environments; The Metabolic and Energy Balance of Men in a Cold Environment; and Field Studies on Cold Adaptation; 375 pages; 119 figures; 13 tables; 1960. Price $6.50.

Group Processes. Fifth Conference, October 12–15, 1958, Princeton, N. J. Edited by BERTRAM SCHAFFNER, M.D., University Seminar on Communications, Columbia University, New York, N. Y. Chairman, HOWARD S. LIDDELL, Behavior Farm Laboratory, Cornell University, Ithaca, N. Y. The contents cover: Experimental Aspects of Pediatrics; The Analysis of Behavior in Terms of Control Systems; and the Cult as a Condensed Social Process; 196 pages; 41 figures; 1960. Price $4.50.